CHARLES UNIVERSITY IN PRAGUE
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Testing of Newly Synthesized Compounds with Constitutive Androstane Receptor (CAR)

Diploma Thesis

Supervisor: Doc. PharmDr. Petr Pávek Ph.D.

HRADEC KRÁLOVÉ 2012 **Ioannis Mouratidis**

I declare that this work is my original author work, which developed by myself. All literature and other sources, which I have used, all are given in the list of used literature and they are quoted in text regularly. The data presented in the work have been used only for the diploma thesis.

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Ioannis Mouratidis

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Thank You,
Ioannis Mouratidis

ABSTRACT

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Interactions of a diverse array of nuclear receptors with numerous isolated compounds (ligands) have been extensively investigated during the last years. The reason of this intense research activity is, of course, the wide therapeutic potential of the nuclear receptors super-family. It is known that they interact with the metabolism and excretion of various compounds, endogenous or exogenous that interfere with the homeostasic mechanisms of the living organisms.

In my study, I tested the activation of a specific orphan nuclear receptor known as constitutive androstane receptor (CAR). I tested 11 structurally different compounds as well as a known inhibitor of CAR (clotrimazole) and an activator (CITCO). To test these interactions between these compounds and CAR, I used the method of the mammalian two-hybrid system, a method where ligand-protein interactions are studied in an environment close to that in vivo. The method is based on the Dual-Luciferase Reporter kit, which made the quantification of the CAR responsive luciferase reporter gene construct activation possible. The results of the transfection were measured by a Genios Plus luminometer which identified the luminescence activity of the cells.

Quantification of luminescence of the compounds showed that compounds 21, 22, 23 and 24 activate efficiently p(ER6)3-luc reporter construct through CAR after 24 hours incubation in MZ-Hep1 cells. Furthermore, compounds 25 and CH3 also activate $p(ER6)_3$-luc but in a lower effect. Cytotoxic/antiproliferative activities of the tested compounds were tested using two approaches including MTS assay. No apparent toxicity was observed.

These data show promising results for further investigation and testing of the activation of CAR in vivo, even if gene reporter assay is a method that efficiently mimics the conditions found in living organisms.

ABSTRAKT

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Testing of newly synthesized compounds with constitutive androstane receptor (CAR)

V minulých letech probíhal intenzivní výzkum hledající ligandy nově objevených nukleárních receptorů. Důvodem této intenzivní výzkumné činnosti je případný široký léčebný potenciál jaderných receptorů. Nukleární receptory se podílí na řízení metabolismu a vylučování různých látek, endogenních i exogenních, a tak řídí homeostázu u živých organismů.

V mé studii jsem testoval aktivaci konkrétního jaderného receptoru známého jako konstitutivní receptor androstane (CAR). Testoval jsem 11 strukturálně různých sloučeniny. Současně jsem použil známý inhibitor CAR klotrimazol a aktivátor CITCO. Pro testování těchto látek jsem použil metodu two-hybrid assay. Tato metoda umožňuje studovat interakce ligand-protein v prostředí blízkému situaci in vivo. Metoda je založena na Dual-Luciferase Reporter kitu, který umožňuje kvantifikaci interakce ligandu s CAR receptorem prostřednictví luciferázového reportérového konstruktu. Výsledná chemiluminiscence byla měřena na Genios Plus luminometru.

Kvantifikace luminiscence testovaných sloučenin ukázala, že látky 21, 22, 23 a 24 aktivují efektivně reportérový konstrukt p$(ER6)_3$-Luc po 24 hodinách inkubace v médiu buněk MZ-Hep1. Kromě toho sloučeniny 25 a CH3 také aktivovaly tento konstrukt p$(ER6)_3$-Luc, ale s nižší efektivitou. Cytotoxická / antiproliferativní aktivita testovaných látek byla testována pomocí dvou přístupů, včetně MTS metody. Žádná zjevná toxicita těchto látek nebyla pozorována.

Tyto údaje ukazují slibné výsledky pro další výzkum a testování aktivace CAR in vivo, jelikož metoda gene reportérové eseje napodobuje podmínky v živých organismech.

Abbreviations list

NR.....nuclear receptor
NHR.....nuclear hormone receptor
DBD.....DNA-binding domain
LBD.....ligand-binding domain
AF-1.....activation function-1
AF-2.....activation function-2
GR.....glucocorticoid receptor
MR.....mineralocorticoid receptor
ER.....estrogen receptor
AR.....androgen receptor
PR.....progesterone receptor
LXR.....liver X receptor
FXR.....farnesoid X receptor
PXR.....pregnane X receptor
CAR.....constitutive androstane receptor
RAR.....retinoid acid receptor
VDR.....vitamin D receptor
RXR.....retinoid X receptor
THR.....thyroid hormone receptor
RE.....receptor element
HNF4α.....hepatocyte nuclear factor 4α
ERR.....estrogen-related receptor
SXR.....steroid and xenobiotic receptor
EcR.....ecdysone receptor
NGF1B.....nerve growth factor 1B
SF-1.....steroidogenic factor 1
FTZ-F1.....“fushi tarazu” factor-1
GCNF.....germ cell nuclear factor
N-CoR.....nuclear receptor co-repressor
SMRT.....silencing mediator for retinoid or thyroid-hormone receptor
CYP.....cytochrome p450
mRNA.....messenger ribonucleic acid
HAT.....histone acetyltransferase
p300/CBP.....p300/CREB-binding protein
CREB.....cAMP response element-binding
TIF2.....transcriptional mediators/intermediary factor 2
SRC-1.....steroid co-activator 1
TRAP.....thyroid hormone receptor-associated proteins
DRIP.....vitamin D receptor interacting protein
ARC.....activator-recruited co-factor
TCDD.....2, 3, 7, 8-tetrachlorodibenzodioxin
PBREM.....phenobarbital-responsive enhancer module
DNA.....deoxyribonucleic acid
MDR1.....multidrug resistance 1
BSEP.....bile salt export pump
MRP1, 2, 3.....multidrug resistance associated protein 1, 2, 3
TH.....thyroid hormone
PB.....phenobarbital
Hsp90.....heat shock protein 90
PP2A.....protein phosphatase 2A
GRIP1.....glucocorticoid receptor interacting protein 1
PGC-1α, -1β.....PPARγ co-activator-1α, -1β

PPRE.....peroxisome proliferator response element
PPARα, β, γ, δ.....peroxisome proliferator-activated receptor α, β, γ, δ
TCPOBOP.....1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene
bHLH-Zip.....basic helix-loop-helix leucine zipper
OATP2.....organic anion-transporting polypeptide
Insig-1, -2.....insulin induced gene-1, -2
TSH.....thyroid stimulating hormone
SCAP.....sterol cleavage-activating protein
HNF4-RE.....HNF4 response element
FOXO1.....forkhead box protein O1
G6P.....glucose-6-phosphate
IRS.....insulin response sequence
LCA.....lithocholic acid
UGT1a.....uridine diphosphate-glucuronosyl transferase 1a
SULT1a1, 2a1.....sulfotransferase1a1, 2a1
SULTn.....sulfotransferase n
CNS.....central nervous system
GSTA1.....glutathione S-transferase A1
COUP-TF.....chicken ovalbuminupstream promoter transcription factor
TR2.....testicular receptor 2
ROR.....RAR-related orphan receptor
ERR.....estrogen-related receptor
AhR.....aryl hydrocarbon receptor
ARNT.....AhR nuclear translocator
AhRR.....AhR repressor
HIV.....human immunodeficiency virus
GST.....glutathione S-transferase
BCRP.....breast cancer resistance protein
CITCO.....6-(4-chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde-O-(3,4-dichlorobenzyl)oxime
HDAC.....histone deacetylase
PXRRE.....PXR response element
CARRE.....CAR response element
ABC transporters.....ATP-binding cassette transporters
HRE.....hormone response elements
DR.....direct repeat
IR.....indirect repeat
SREBP1.....sterol regulatory element binding protein 1
HMG-CoA.....hydroxy methyl glutaryl-coenzyme A
CCRP.....CAR cytoplasmic retention protein
OARE.....okadaic acid response element
HepG2.....liver hepatocellular cells
SMC1.....structural maintenance of chromosomes 1
OA.....okadaic acid
PBRE.....phenobarbital response element
RARE.....retinoic acid response element

Contents

1. Introduction

Nuclear receptors (NRs) are ligand-activated transcription factors that transform extra- and intracellular signals into cellular responses by activating the transcription of NR target genes (Honkakoski et al. 2000). What is more, they share a common evolutionary history and have acquired similar sequence characteristics at the protein level (di Masi et al. 2009). NRs regulate the expression of genes whose products are important for embryonic development, maintenance of differentiated cellular phenotypes, metabolic disorders such as obesity, type 2 diabetes, hyperlipidemia, hypertension, atherosclerosis and cell death. Dysfunction of nuclear receptor signaling cause serious problems in the organism leading to proliferative, reproductive and metabolic diseases such as cancer, infertility, obesity and diabetes (Nakata et al. 2006).

2. Theoretical focus on Nuclear Receptors and CYP450 enzymes

2.1 Nuclear receptor super-family: structure, function, sub-families, evolution

The nuclear receptor superfamily is composed of a diverse array of transcription factors, which include nuclear hormone receptors (NHRs) and orphan nuclear receptors. NHRs are receptors with identified hormonal ligands (Olefsky 2001). Orphan nuclear receptors (NRs) are composed of gene products that are structurally similar to nuclear hormone receptors but lack known physiological ligands (Xie et al. 2001).

The following figure shows that all of the nuclear receptors have common structural features. They include a central DNA binding domain (DBD), which is the most highly conserved domain and attached to it are two zinc finger modules, responsible for targeting the receptor to highly specific DNA sequences that create a response element. Next, the ligand binding domain (LBD) is situated in the C-terminal half of the receptor and accepts specific hormonal and non-hormonal ligands directing specificity to the biologic response. These receptors contain variable N-terminal and C-terminal domains. N-terminal region (A/B) contains ampF-I activation function (AF-1) and also a variable length hinge region between the DBD and LBD (Olefsky 2001). On the other hand ligand-dependent activation function 2 (AF-2) is located at the carboxy-terminal end of the LBD (Nakata et al. 2006).

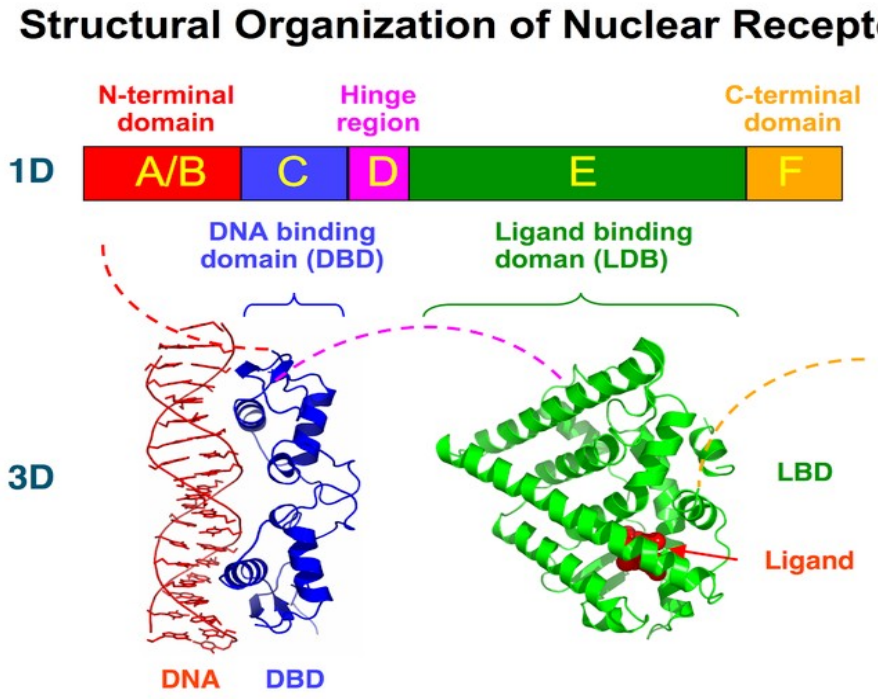


Fig. 1* Structural organization of Nuclear receptors

Top – Schematic 1D amino acid sequence of a nuclear receptor.
Bottom – 3D structures of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor. The structures shown are of the estrogen receptor. Structures of N-terminal domain (A/B), hinge region (D), and C-terminal domain (F) are represented by red, purple, and orange dashed lines, respectively. (Created using PyMol software)

***from www.en.wikipedia.org/wiki/Nuclear_receptor**

According to Manglesdorf et al., there exist four categories of nuclear receptors in which Class 1 receptors include the known steroid hormone receptors, which function as homodimers binding to half-site RE inverted repeats. The Class 1 involves glucocorticoid (GR), mineralocorticoid (MR), estrogen (ER), androgen (AR) and progesterone (PR) receptors (Nakata et al. 2006). Whereas, class 2 receptors exist as heterodimers with RXR and function in a ligand-dependent manner. They involve receptors for fatty acids (PPARs), oxysterols (LXRs), bile acids (FXR) and xenobiotics (PXR, CAR) (Nakata et al. 2006). The other two classes include orphan receptors, which function as homodimers binding to direct RE repeats (Class 3) or monomers binding to single site REs (Class 4). Hence, nuclear receptors can exist as monomeric proteins and homo- or heterodimers

where each partner binds to specific RE sequences that exist as half-sites separated by variable length nucleotide spacers between direct or inverted half-site repeats (Olefsky 2001).

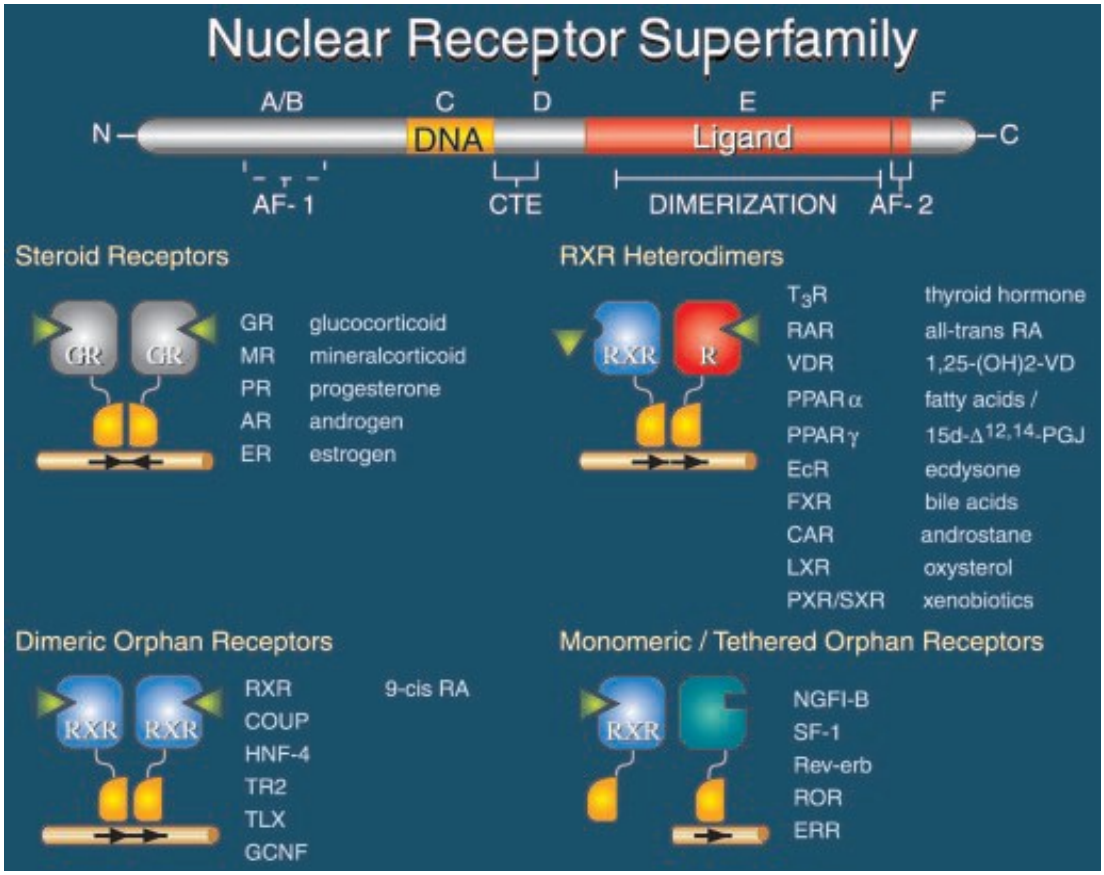


Fig. 2* Structure/function organization of nuclear receptors

This picture shows a diverse class of receptors that function as:

(I) Homodimers (Class 1 of steroid receptors)
(II) Heterodimers with RXR (Class 2)
(III) Homodimers which bind to direct receptor elements (Class 3 of orphan receptors)
(IV) Monomers which bind to single site receptor elements (Class 4 of orphan receptors)

***from Olefsky 2001**

Nuclear receptors create a superfamily of phylogenetically related proteins, with 21 genes in the complete genome of the fly Drosophila melanogaster, and 48 in humans. (Robinson-Rechavi et al. 2003). In addition, NRs share a common evolutionary history as revealed by their conserved structure and by their high degree of sequence conservation. The phylogenetic analysis of the NR super-family brought to its sub-division into six sub-families of variable size: (i) the large subfamily I includes the thyroid hormone receptors (THRs), the retinoic acid receptors (RARs), the peroxisome proliferator-activated receptors (PPARs), the Vitamin D receptor (VDR), the ecdysone receptor, as well as numerous orphan receptors such as CAR and PXR; (ii) the sub-family II includes RXR, the hepatocyte nuclear factor 4 (HNF4), and the chicken ovalbumin upstream promoter transcription factor (COUP-TF); (iii) the steroid receptor sub-family III includes the estrogen receptors (ERs), the estrogen-related receptors (ERRs), the progesterone receptor (PR), the glucocorticoid receptors (GRs), the mineralocorticoid receptor (MR), as well as the androgen receptors (ARs); (iv) the sub-family IV includes the nerve growth factor inducible I-B group of orphan receptors (NGF1B); (v) the sub-family V contains the steroidogenic factor-1 (SF-1) and the Drosophila melanogaster ‘‘fushi tarazu” factor-1 receptor (FTZ-F1); and (vi) the smallest of all, sub-family VI, contains only the germ cell nuclear factor-1 receptor (GCNF) (di Masi et al. 2009, Laudet et al. 1997, Owen and Zelent 2000).

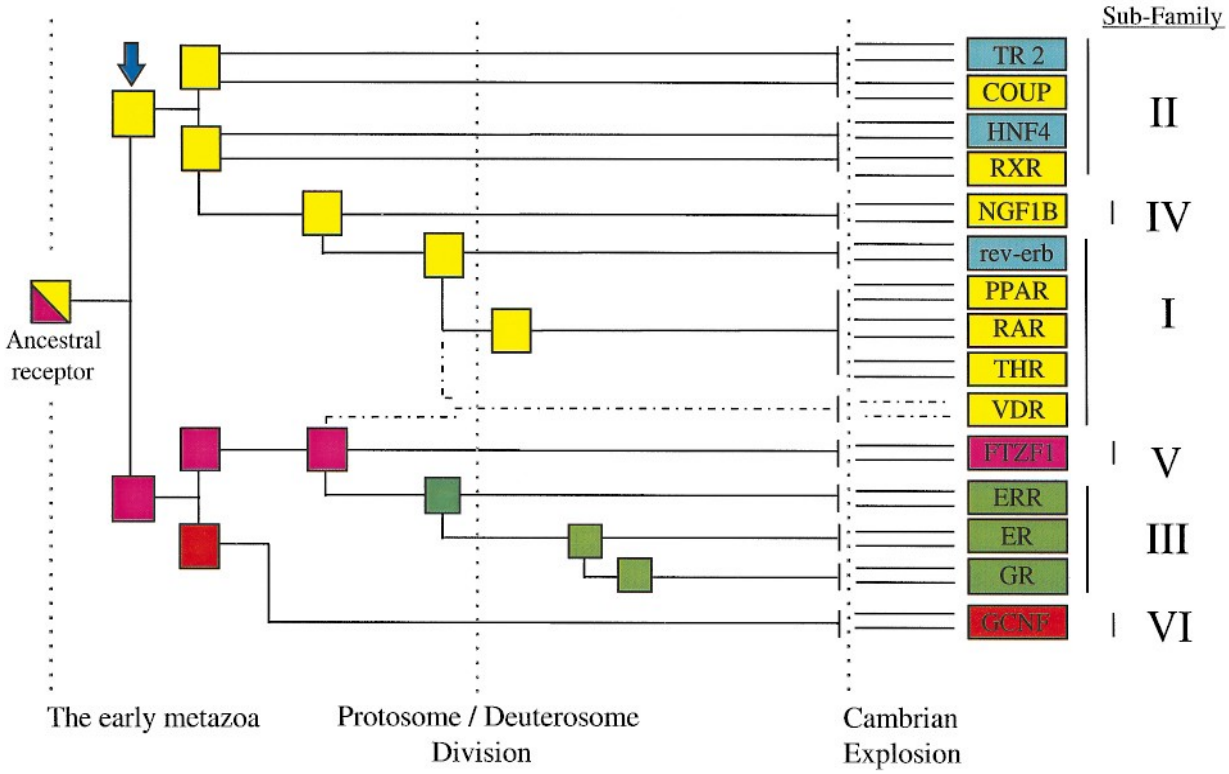


Fig. 3* Evolutionary aspects of the NR super-family

A hypothetical evolutionary path that the first NR could have taken in the early metazoan and how it might have diverged into the six sub-families that are known today.

***from Owen and Zelent 2000**

According to the figure shown above, the NR ancestor in the early metazoan was a common ancestor of sub-family II (yellow box) and V (pink box). Heterodimerisation was acquired early in the diversification of the family (blue arrow) and was either conserved (yellow box) or lost (blue box). The sub-family V ancestor gave rise to sub-family VI (red box) and to a receptor, which through further diversification of the ligand-binding domain gave rise to sub-family III (green box). Dashed lines represent the possible origin of a chimeric VDR. The vertical green dashed lines show the time period of the first metazoan, the protosome-deuterosome split and the Cambrian explosion which saw the HOX gene cluster quadruple and the appearance of numerous NR paralogues that are known today in vertebrates (Owen and Zelent 2000).

Nuclear receptors commonly act in three steps: repression, de-repression and transcription activation. Repression is characteristic of the apo-nuclear receptor, which

recruits a co-repressor complex with histone de-acetylase activity (Robinson-Rechavi et al. 2003). Indeed, some ligand-free NRs interact with co-repressors such as N-CoR and SMRT to suppress gene transcription by the recruitment of histone deacetylases (Honkakoski et al. 2000). Next, de-repression occurs following ligand binding, which dissociates this complex and recruits a first co-activator complex, with histone acetyltransferase (HAT) activity resulting in chromatin decondensation (Robinson-Rechavi et al. 2003). Ligand binding induces great structural changes in the folding of the LBD, with AF-2 being repositioned so as to form a hydrophobic patch that is accessible to common co-activators and co-integrators such as SRC-1, p300/CBP and TIF2. Co-activators bind to ligand-bound NRs through their LXXLL repeats and they either possess intrinsic histone acetyltransferase activity or recruit additional histone acetyltransferases that derepress the chromatin to activate transcription (Honkakoski et al. 2000). Finally, the HAT complex dissociates and a second coactivator complex is assembled (TRAP/DRIP/ARC), which may establish contact with the basal transcription machinery, and thus facilitates transcription activation of the target gene. However, this mechanism is not general, since some nuclear receptors may function as activators without a ligand, whereas others are unable to interfere with the target gene promoter in the absence of ligand (the 'repression' step) (Robinson-Rechavi et al. 2003).

2.2 CYP450 enzymes: families, induction, functions, drug interactions

Humans have 57 genes and more than 58 pseudogenes expressing 18 families of cytochrome P450 genes and 43 sub-families. Genes encoding CYP enzymes, and the enzymes themselves, are shown with the abbreviation "CYP", followed by an Arabic numeral that indicates the gene family, then a capital letter indicating the subfamily, and finally other numerals for the individual gene. Members of the heme-containing monooxygenases super-family are involved in phase I of xenobiotic biotransformation, cholesterol biosynthesis, endogenous compound and steroid hormone metabolism, and steroidogenesis in eukaryotic organisms. Fifteen human CYPs are mainly involved in xenobiotic metabolism. All of them being from CYP1, CYP2 and CYP3 families (Pavek et al. 2008).

Families CYP1, CYP2 and CYP3 play a key role in phase I of xenobiotic biotransformation. The main members of human CYP1A family are CYP1A1, CYP1A2

and CYP1B. CYP1A1 is one of the most important detoxification enzymes because of its broad substrate specificity and wide distribution throughout the body. Nevertheless, CYP1A1 can also produce highly carcinogenic metabolites by the oxidation of polycyclic aromatic hydrocarbons. CYP1B1 gene is expressed differentially between tissues, with the highest concentration of mRNA detected in extrahepatic tissues such as heart, brain, uterus, lung, skeletal muscle and kidney. CYP1B metabolizes a variety of polycyclic aromatic hydrocarbons and is involved in the metabolism of endogenous steroids, arachidonate, retinol and retinal, and melatonin. CYP1A2 is a hepatic enzyme that metabolizes several clinically relevant drugs such as caffeine, zolmitriptan, tizanidine, and tacrine, which are not at the same time substrates of CYP1A1. The most important genes of CYP2 family that take part in the xenobiotic metabolism of clinically relevant drugs and alcohol are CYP2A6, CYP2C8, CYP2B6, CYP2C19, CYP2C9, CYP2D6, CYP2E1, and CYP2F1. CYP2C9 is the second most abundantly expressed CYP in human liver and intestine. There was estimated that CYP2C9 metabolizes about 16% of clinically prescribed drugs. Finally, CYP3 family comprises only four members in humans: CYP3A5, CYP3A4, CYP3A7 and CYP3A43. CYP3A4 is the most important isoform, being implicated in the metabolism of more than 50% of xenobiotics. Consistently, CYP3A4 is highly expressed in principal sites of drug disposition including the liver and small intestine (Pavek et al. 2008).

Table 1* Nomenclature for the main families of Cytochrome P450 involved in xenobiotic metabolism

Family	Main characteristics	Members	Names
CYP1	Found in liver and extra-hepatic tissues. Important in drug metabolism, steroid metabolism (mainly estrogens), and heme metabolism. Induced by polycyclic aromatic hydrocarbons	3 sub-families 3 genes 1 pseudogene	CYP1A1, CYP1A2, CYP1B1
CYP2	Found in liver. Important in the metabolism of many classes of drugs. Induced by ethanol and acetone.	13 sub-families 16 genes 16pseudogenes	CYP2A6, CYP2A7, CYP2A13, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2F1, CYP2F2, CYP2R1, CYP2S1, CYP2U1, CYP2W1
CYP3	Found in fetal and adult liver, in gastrointestinal tract, in kidney and in placenta. Important in the metabolism of majority of drugs and steroids and many hydrophobic substrates. Induced mainly by glucocorticoids and phenobarbital.	1 sub-family 4 genes 2 pseudogenes	CYP3A4, CYP3A5, CYP3A7, CYP3A43
CYP4	Found in liver and kidney. Important in metabolism of free fatty acids, in the catabolization of leukotrienes and prostanoids, in the synthesis of bioactive metabolites from arachidonic acid and in xenobiotic metabolism	6 sub-families 11 genes 10 pseudogenes	CYP4A11, CYP4A22, CYP4B1, CYP4F2, CYP4F3, CYP4F8, CYP4F11, CYP4F12, CYP4F22, CYP4V2, CYP4X1, CYP4Z1
CYP7	Found in liver and brain. Important in the synthesis of bile acids. Also, in neurosteroid metabolism and sex hormone synthesis	2 sub-families 2 genes	CYP7A1, CYP7B1

***from di Masi 2009 with some structural changes**

Xenobiotic chemicals such as drugs and environmental chemicals, are absorbed by the intestine and transported to the target sites (CYP enzymes, NRs, membrane receptors, transporters) (Nakata et al. 2006). The induction of cytochrome P450 (CYP) enzymes by xenobiotics is a major concern because of the increased metabolism of pharmaceutical drugs and endogenous substrates. The oxidative metabolism of pharmaceuticals to more polar metabolites is an important way to eliminate the xenobiotics (Tompkins et al. 2007). CYP450 enzymes are named like this because they bind to membranes within a cell (cyto) and include a heme pigment in their structure (chrome and P) that absorbs light at a wavelength of 450 nm when exposed to carbon monoxide. There are more than 50 CYP450 enzymes, but the CYP1A2, CYP3A4, CYP2C9, CYP2C19, CYP2D6, and CYP3A5 metabolize approximately 90% of all the drugs. These enzymes are predominantly expressed in the liver, but they also reside in the small intestine (reducing drug bioavailability), placenta, lungs and kidneys. What is more is that a specific gene encodes each CYP450 enzyme. Every person inherits one genetic allele from each parent. Alleles are classified as “wild type” or “variant,” with wild type occurring most commonly in the general population. An “extensive” metabolizer has received two copies of wild type alleles. Polymorphism happens when a variant allele takes the place of one or both wild type alleles. Variant alleles usually encode a CYP450 enzyme with reduced or no activity. Persons with two copies of variant alleles are “poor” metabolizers, on the contrary those with one wild-type and one variant allele have reduced enzyme activity. Finally, a number of persons inherit multiple copies of wild-type alleles, which results in excess enzyme activity. This phenotype is called an “ultrarapid” metabolizer (Lynch et al. 2007).

Predominantly, induction of CYPs occurs by a process involving de novo RNA and protein synthesis that has been investigated in studies using transcription and translation inhibitors. The induction of many CYPs occurs by a similar mechanism, where ligand activation of major receptor transcription factors including pregnane X receptor (PXR), constitutive androstane receptor (CAR), aryl hydrocarbon receptor (AhR) and others, leads to increased transcription (Tompkins et al. 2007).

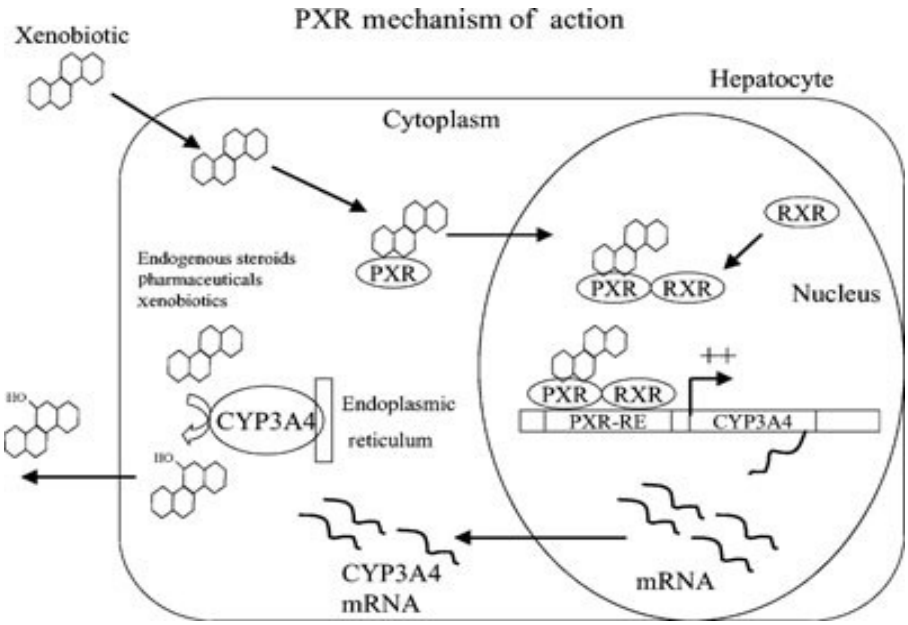


Fig. 4* Xenobiotic-induction of CYP3A4 gene by PXR

A xenobiotic acts as a ligand and binds with PXR inside the cell. The complex enters the nucleus, forms a heterodimer with RXR and binds to the corresponding response element initiating the transcription of CYP3A4 gene.

***from T. Lynch et al. 2007**

Pregnane X receptor (PXR, NR1I2) activation mediates the inducible expression of CYP3A in mice. Biochemical studies suggest that PXR and CAR bind the same or overlapping enhancer elements within the promoters of CYP3A and CYP2B genes in liver cells in a competitive manner. The pregnane X receptor (PXR/NR1I2) responds to a wide range of pharmaceutical agents, steroids, environmental contaminants, and toxic bile acids. It binds to xenobiotic response elements, and forms a heterodimer with the 9-cis retinoic acid receptor (RXR). Thus, PXR regulates the variation in expression of a gene. In fact, PXR is a key regulator of CYP3A expression in mammalian liver and small intestine. PXR regulates also a variety of genes that are important in xenobiotic metabolism, including those encoding proteins in phase I metabolism (oxidation), phase II metabolism (conjugation), and phase III transport (elimination). All of these gene products take part in the solubilization or active excretion of drugs and other xenobiotics (Staudinger et. al. 2003). Equally important, the constitutive androstane receptor (CAR/NR1I3), which is discussed in details during the next chapters, is a xenobiotic-sensing receptor that is

capable of recognizing structurally diverse compounds. In brief, it was originally characterized receptor that activates a retinoic acid response element (RARE) without the need of a ligand. As a heterodimer with RXR, CAR regulates the phenobarbital-mediated induction of UGT1A1, CYP2B6, CYP3A4, and CYP2C9 (Nakata et al. 2006).

CYP enzymes, especially members of the CYP 1–4 families, are very important for xenobiotic detoxification and survival of organisms. Among CYP enzymes, the CYP3A and -2B isoenzymes are of particular pharmacological and toxicological significance. For example, the human CYP3A4 enzyme alone metabolizes approximately 50–60% of clinical drugs as well as nutraceuticals and herbal medicines. Moreover, an additional 25-30% of these compounds are metabolized by the CYP2B isoenzymes. The combined metabolic ability of CYP3A and -2B, coupled with their inducibility by xenobiotic substrates, creates a molecular basis for many clinical drug-drug interactions (Xie et al. 2001).

Many drug interactions could result in the alteration of CYP450 metabolism. Drugs interact with the CYP450 system in many ways. Drugs can be metabolized by only one CYP450 enzyme (i.e., metoprolol by CYP2D6) or by multiple enzymes (i.e., warfarin by CYP1A2, CYP2D6, and CYP3A4). Drugs that cause CYP450 metabolic drug interactions are indicated as either inhibitors or inducers. Inhibitors block the metabolic activity of one or more CYP450 enzymes. The degree at which an inhibitor affects the metabolism of a drug depends upon factors such as the dose and the ability of the inhibitor to bind to the enzyme. Inhibitory effects usually occur immediately. Additionally, a drug can both inhibit and be metabolized by the same enzyme (e.g., erythromycin), or it can inhibit one enzyme and be metabolized by another one (e.g., terbinafine). Drugs may be intentionally combined to take advantage of CYP450 inhibition. Ritonavir, a protease inhibitor and potent CYP3A4 inhibitor, is added to lopinavir to increase serum levels in patients with human immunodeficiency virus (HIV). Inducers increase CYP450 enzyme activity by increasing enzyme synthesis. In contrast to metabolic inhibition there is usually a delay before enzyme activity increases that depends on the half-life of the inducing drug. A decrease in the concentration of a drug metabolized by CYP2C9 can occur within 24 hours after the initiation of rifampin (Rifadin), an inducer with a short half-life. However, a decrease in the concentration of this drug can occur up to one week after the initiation of phenobarbital, an inducer with a very long half-life. A drug also may be metabolized by the same CYP450 enzyme that it induces. Carbamazepine, a potent enzyme inducer, must first

be taken at a low dose and then increased at weekly intervals as its half-life gradually decreases over time(T. Lynch et al. 2007).

Table 2* Significant Cytochrome P450 Enzymes and Their Inhibitors, Inducers, and Substrates

Enzyme	Potent inhibitors	Potent inducers	Substrates
CYP1A2	Amiodarone , cimetidine, ciprofloxacin , fluvoxamine	tobacco	Caffeine, clozapine, theophylline
CYP2C9	Amiodarone, fluconazole, fluoxetine, metronidazole, ritonavir, trimethoprim/sulfamethoxazole	Carbamazepine, phenobarbital, phenytoin, rifampin	Carvedilol, celecoxib, glipizide, ibuprofen, irbesartan, losartan
CYP2C19	Fluvoxamine, isoniazid (INH), ritonavir	Carbamazepine, phenytoin, rifampin	Omeprazole, phenobarbital, phenytoin
CYP2D6	Amiodarone, cimetidine, diphenhydramine, fluoxetine, paroxetine, quinidine, ritonavir, terbinafine	No significant inducers	Amitriptyline, carvedilol, codeine, donepezil, haloperidol, metoprolol, paroxetine, risperidone, tramadol
CYP3A4 and CYP3A5	Clarithromycin, diltiazem, erythromycin, grapefruit juice, itraconazole, ketoconazole, nefazodone, ritonavir, telithromycin, verapamil	Carbamazepine, *Hypericum perforatum* (St. John's wort), phenobarbital, phenytoin, rifampin	Alprazolam, amlodipine, atorvastatin, cyclosporine, diazepam, estradiol, simvastatin, sildenafil, verapamil, zolpidem

***from T. Lynch 2007 with some structural changes**

The ability of compounds to induce CYPs can be investigated in tissue culture models by transient transfections using immortalized cells expressing relevant receptors and CYP promoter–reporter plasmids. Alternatively, primary cultures of rodent and human hepatocytes have been extensively used to find out whether compounds can induce CYP mRNA, protein, and metabolic activity. Meanwhile, humanized transgenic mice, where the mouse gene has been disrupted and the human CYP gene has been introduced, express only human CYPs and have been very useful in the studies of human CYP450`s gene induction, metabolism, and toxicity. Our recent understanding of the receptors critical action in the induction of CYPs has also lead to the development of many new transgenic mouse models in which the mouse receptor gene has been replaced with the human receptor. These transgenic models include humanized PXR, AhR, CAR, and PPARα mice, which have been very useful for the study of the mechanisms of induction (Tompkins et al. 2007).

2.3 Regulation of CYP450 genes by Nuclear Receptors

The gene superfamily of nuclear receptors includes a number of ligand-dependent and ligand-independent transcription factors that are usually characterized by C-terminal ligand binding domain and a zinc finger DNA binding domain as shown in Fig. 1. Nuclear receptors were prime candidates for mediating hepatic drug induction for many reasons. First, their ligands are normally small and lipophilic, properties very similar to those of xenobiotic and endobiotic inducer compounds such as steroids, bile acids, or fatty acids. Second, nuclear receptors bind to DNA elements consisting of repeats of hexamers in different kind of arrangements such as those found in drug-responsive enhancers of P450s. Third, the tissue-specific expression of a subset of nuclear receptors is identical to the tissue specificity of drug induction. Finally, closely related members of the nuclear receptor subfamilies NR1I and NR1H play major roles in many physiological processes where P450s are involved. These include steroid, vitamin D, lipid, cholesterol, or bile acid biosynthesis and metabolism (Handschin et al. 2003).

2.3.1 Xenobiotic-, estrogen- and retinoid-metabolizing CYP1 forms

The aryl hydrocarbon receptor (AhR), AhR nuclear translocator (ARNT) and AhR repressor (AhRR) are members of the bHLH-PAS transcription factor (TF) superfamily (K. Nakata et al. 2006). Polyaromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induce CYP1A, CYP1B, GST, UGT1A and AhRR genes through binding to the aryl-hydrocarbon receptor (AhR), translocation of the ligand-bound AhR into the nucleus, and association of the AhR with its dimerization partner, AhR nuclear translocator (ARNT). Next, the AhR/ARNT complex binds to xenobiotic response elements and initiates the CYP gene transcription in a different kind of tissues. The induction can be regulated by the protein kinase C pathway (Honkakoski et al. 2000). The AhR is then excreted to the cytosol and degraded by 26S proteasome pathway. AhRR suppresses AhR transcriptional activity by competing with AhR for dimerizing with ARNT and binding to the DRE site of target genes (Pavek and Dvorak 2008).

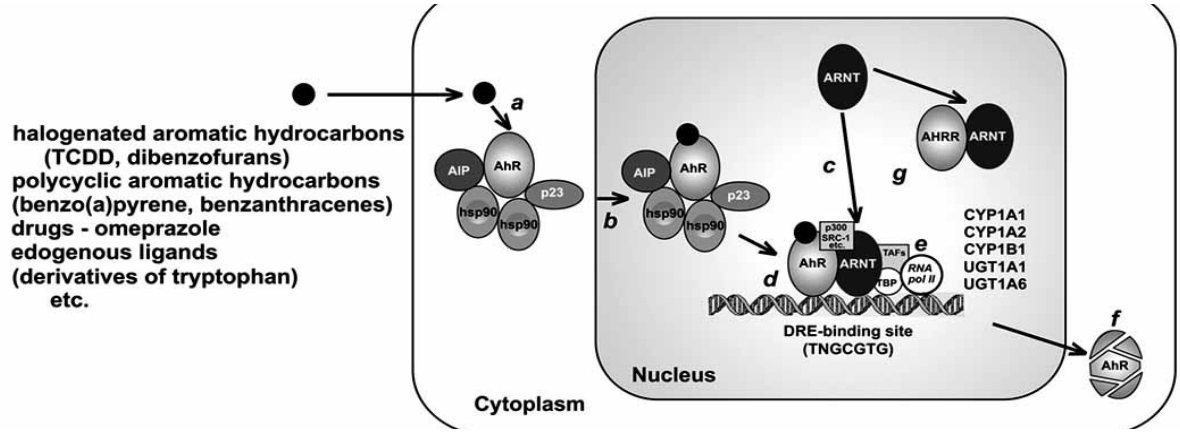


Fig 5* The ligand-activated AhR signal transduction pathway

Expression of a variety of CYP1 forms (shown in the figure), that are involved in the metabolism of a diverse array of drugs, occur by the induction of Aryl hydrocarbon Receptor (AhR).

***from Pavek and Dvorak 2008**

Estrogen receptors (ERs), ERα (NR3A1) and ERβ (NR3A2), are parts of the steroid hormone receptor superfamily, and ligand-dependent transcription factors. ERs generally

bind as homodimers to hormone REs that consist of palindromic arrangements. ERs play a multitude of crucial roles not only in development, reproduction, homeostasis and immune functions but also in different types of cancer (breast, ovarian, colorectal etc), osteoporosis, cardiovascular disease, neurodegenerative diseases and obesity (K. Nakata et al. 2006). Maximal CYP1A1 induction depends on the presence of ERα in some cell lines. The ERα may act indirectly on cell type specific factors, because an acute treatment with anti-estrogens did not influence CYP1A1 or CYP1B1 expression in carefully selected ERα-positive cells. Both genes were induced by TCDD, regardless of the ERα status. A role for ERβ that can be activated by ERα ligands or for ER-related receptors should also be considered. In conclusion, ERα seems to exert its effects on CYP1 gene expression indirectly (Honkakoski et al. 2000).

In keratinocytes, retinoic acid (RA) has been reported either to down-regulate or up-regulate CYP1A1 gene expression. The CYP1A1 gene binds an unusual DR4 element that conferred a modest RA-dependent increase in reporter-gene activity. In hepatocytes, retinoids had small effect on CYP1A1 or CYP1A2 mRNAs, while RXR- and RA receptor (RAR)- selective ligands lowered hepatic CYP1A2 in intact animals (Honkakoski et al. 2000).

2.3.2 Steroid- and fatty acid-metabolizing CYP2B, CYP3A and CYP4A forms

Constitutive androstane receptor (CAR) was initially isolated and shown to bind a DR-5 type of retinoid acid response element (RARE) in a ligand-independent manner (Honkakoski et al. 2000). As a heterodimer with RXR, CAR mediates the phenobarbital induction of CYP2B6, CYP3A4, UGT1A1. PB and 5β-pregnane are CAR activators and clotrimazole, progesterone and androstenol are CAR deactivators. CAR is a xenobiotic-sensing receptor that is capable of recognizing structural various compounds (Nakata et al. 2006). The identity of CAR as a xenobiotic receptor was first identified by the ability of selective androstane metabolites to inhibit its constitutive activity. Its role in positive xenobiotic regulation was suggested when CAR was shown to activate the Phenobarbital response element situated in promoters of PB-inducible CYP2B genes. Consequently, this activation was found to be potentiated by PB and its derivatives such as TCPOBOP (Xie et al. 2001). PB-responsive primary hepatocyte cultures, in situ DNA injection techniques, and the availability of active and inactive derivatives of a powerful inducer, 1,4-bis-[2-

(3,5-dichloropyridyloxy)]benzene facilitated the discovery of PB-responsive DNA elements (PB-responsive enhancer module; PBREM). PBREM-like elements are situated approximately at 2300 bp in rat CYP2B2 and mouse CYP2B10 genes and at 1700 bp in human CYP2B6 (Honkakoski et al. 2000).

Human orphan receptor SXR (steroid and xenobiotic receptor) and its rodent ortholog PXR (pregnane X receptor) were shown to be activated by different kinds of xenobiotic compounds and steroids that were known to induce hepatic and intestinal CYP3A activity. Moreover, SXR and PXR are able to bind to the IR-6 and DR-3 xenobiotic response elements situated in the promoter regions of the human or rodent CYP3A (Xie 2001). Its ability to bind to both IR6 and DR3 motifs, and their coexpression with CYP3A isoforms strongly indicated that PXR can regulate CYP3A genes (Honkakoski et al. 2000). The CYP3A enzyme is responsible for metabolizing and clearing more than 50% of clinically prescribed drugs. PXR also mediates drug efflux by inducing the expression of the Multidrug resistance 1 (MDR1) gene, which encodes the P-glycoprotein (MDR1/ABCB1). In humans, PXR also mediates CYP3A7, CYP2B6 as well as a variety of other proteins such as the bile salt export pump (BSEP/ABCB11), multidrug resistance associated proteins 1,2,3 (MRP 1,2,3/ABCC1,2,3), and breast cancer resistance protein (BCRP/ABCG2) (Nakata et al. 2006).

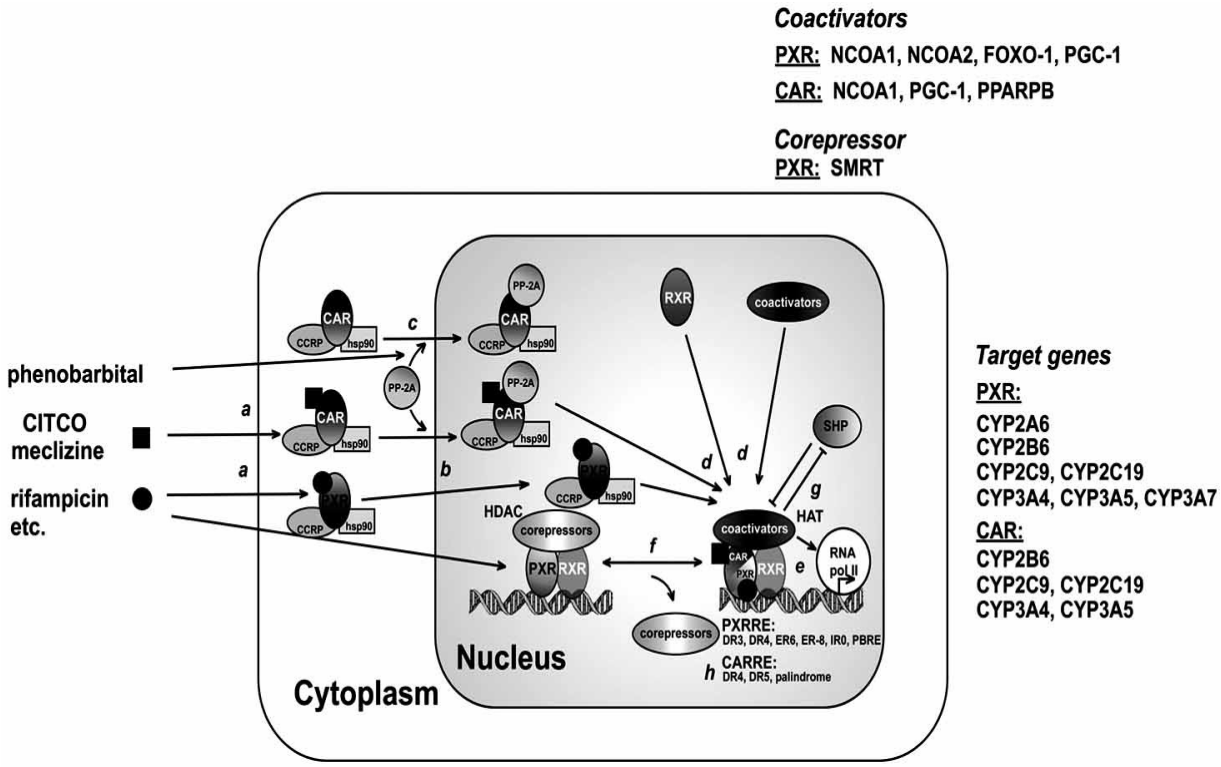


Fig. 6* The PXR and CAR nuclear receptor transactivation pathway

Transactivation of CAR and PXR by ligands such as CITCO and TCPOBOP can lead to expression of a number of CYP genes that play major role in the metabolism and excretion of both endogenous and exogenous compounds

***from Pavek and Dvorak 2008**

The liganded nuclear receptor binds to RXR nuclear receptors forming PXR/RXR or CAR/RXR heterodimers, which recruits coactivators and bind to regulatory regions (PXRRE or CARRE) of a diverse array of target genes listed in the picture above. Coactivators consist of histone acetyltransferase activity (HAT) that induces chromatin decompactation, which then promotes gene activation. PXR is also associated in a corepressor complex in the absence of ligand, containing SMRT (NCOR2) and recruits histone deacetylases (HDACs). Deacetylation of histones leads to chromatin compaction and transcriptional repression of a gene. On the other hand, ligand binding to PXR nuclear receptor causes release of the corepressors, recruitment of a coactivator complex and finally transcriptional activation of gene expression (Pavek et al. 2008).

Phthalate esters, lipid-lowering fibrate drugs, and other chemicals share the property of being peroxisome proliferators. The receptor activated by peroxisome proliferators is

PPARa. Two other PPAR isoforms (PPARδ and PPARγ), with distinct tissue specificities and functions, are also known (Honkakoski et al. 2000). The peroxisome proliferator-activated receptors α, γ, δ (PPARα, γ, δ/NR1C1, 2, 3) are a family of fatty acid-activated TFs and dietary lipid sensors, which regulate lipid homeostasis and cellular differentiation. They bind to DR1 hormone-response elements (HREs) as heterodimers with RXR, where DR1 is a direct repeat with 1 bp spacing (Nakata et al. 2006).

PPARα was initially found to respond to hypolipidemic drugs, such as fibrates. However, it was discovered that fatty acids act as their natural ligands. PPARα induces the expression of the fatty acid metabolizing enzymes including several isoforms of the CYP4A gene subfamily. What is more is that in human macrophages, a PPARa activator induces the expression of the ABC transporter ABCA1 gene which controls apoAI-mediated cholesterol efflux from macrophages. PPARγ is the key regulator of adipogenesis, but also plays an important role in cellular insulin sensitivity, differentiation, atherosclerosis and cancer. PPARδ increases cholesterol efflux from cells, through an increase in the expression of cholesterol transporter ABCA1 (Nakata et al. 2006).

2.3.3 Vitamin D- and Retinoid-metabolizing CYP forms

Vitamin D is a precursor for biologically active 1α,25-dihydroxyvitamin D_3, which has a crucial role in regulation of calcium homoeostasis and cell differentiation. The 1α,25-dihydroxyvitamin D_3 binds to the vitamin D receptor (VDR), VDR/RXR heterodimer then binds to DR3 elements present in target genes and it initiates their transcription via AF-2- and coactivator- dependent mechanisms. 1α,25-Dihydroxyvitamin D_3 is formed by 25-hydroxylation in liver and CYP27A1 catalyzes the reaction. Additionally, CYP24 inactivates vitamin D to calcitroic acid (Honkakoski et al. 2000).

Retinoids are a group of vitamin A derivatives that have significant effects on cell growth and differentiation. They apply two distinct NR signalling pathways, the RARs and the RXRs, which bind to DR2/DR5 and DR1 response elements respectively. CYP2B and CYP2C isoforms primarily convert retinoids into presumably inactive 4-hydroxy derivatives . Novel 4- oxo acid and aldehyde derivatives are also powerful and abundant RAR ligands, and they can be produced by CYP1A2. A novel RA 4-hydroxylase gene, CYP26, is highly expressed in liver and brain and present in several cell lines. CYP26

mRNA is expressed by RA via action of RARγ/RXRα heterodimers through not-known yet DNA elements (Honkakoski et al. 2000).

2.3.4 Cholesterol-metabolizing CYP forms

The cholesterol metabolism is regulated at the biosynthetic pathway by oxysterols that prevent activation of SREBP family of regulators. The biosynthetic pathway involves only one CYP enzyme, called lanosterol 14a-demethylase CYP51. CYP51 produces so-called meiosis-activating sterols that can activate LXRα and possibly the ubiquitously expressed LXRβ as well (P. Honkakoski et al. 2000). Moreover the liver X receptors LXRα (NR1H3) and LXRβ (NR1H2), which form heterodimers with RXR, are bound and activated by naturally occurring oxysterols and other small lipophilic agents. LXRs behave as cholesterol sensors to mediate the transcription of gene products that control intracellular cholesterol homeostasis through biosynthesis, transport and catabolism. LXRα is activated by oxysterols and initiates the conversion of cholesterol to bile acids by inducing CYP7A1 transcription (K. Nakata et al. 2006). LXRα could be activated by several oxysterols, such as the 24(*S*)-OH derivative. LXRα can bind to, and activate, the CYP7A promoter via the DR4 motif at 74 bp, while the related LXRβ was not able to bind to DR4 well. The DR4 motif is situated within the proximal of the two elements crucial for the bile acid response (Honkakoski et al. 2000). To conclude, LXRα also regulates different kind of genes involved in cholesterol and/or lipid homeostasis including ABC transporters (ABCA1, ABCG1 etc.) (Nakata et al. 2006).

Bile acids such as chenodeoxycholic acid have now been established as ligands for FXR, an NR that is present mainly in liver and kidney. The farnesoid X receptor (FXRα/NR1H4) is member of the orphan receptors, and forms a heterodimer with RXR.In the enterohepatic system, FXR functions as a bile acid sensor and regulates bile acid synthesis and recirculation for the protection of the body from elevated bile acid concentration. FXR suppresses the expression of CYP7A and CYP8B genes, which are rate-limiting enzymes for the production of bile acids. (Nakata et al. 2006).

3. Theoretical focus on constitutive androstane receptor (CAR)

The constitutive androstane receptor (CAR, NR1I3) is member of the nuclear receptor superfamily that mediates the expression of genes involved in the metabolism of hormones and xenobiotics. CAR operates as a heterodimer with the retinoid X receptor (RXR, NR2B) and is classified in a subfamily species called orphan receptors, that includes the pregnane X receptor (PXR, NR1I2) and the vitamin D receptor (VDR, NR1I1) (Xu et al. 2004). CAR differs from PXR and VDR in having high constitutive activity in the absence of ligand. CAR genes have so far been found only in mammals. The high constitutive activity of CAR means that some compounds act as "inverse agonists" and decrease the level of constitutive activation while other function as agonists and increase even more the activation (Reschly et al. 2006).

3.1 Structure and activation of human CAR gene and protein

hCAR is the product of the NR1I3 gene, which is sited on chromosomes 1, locus 1q23, and spans approximately 8.5 kilobases (kbs). The NR1I3 gene consists of 8545 base pairs (bps), and contains 9 exons separated by 8 intronic regions (di Masi et al. 2009).

According to di Masi et al. twenty-two unique hCAR splice variants, containing various combinations of splicing, have been identified. Although some CAR mRNAs originate from a single splicing event, the majority of the CAR transcript isoforms arise from multiple alternative splice events in a variety of combinations. Some CAR splice variants are unlikely to encode functional proteins because they have premature termination codons originating from nonsense mutations, and they are rapidly degraded. The rest of the alternative CAR mRNAs could encode unique CAR proteins as shown in figure 7.

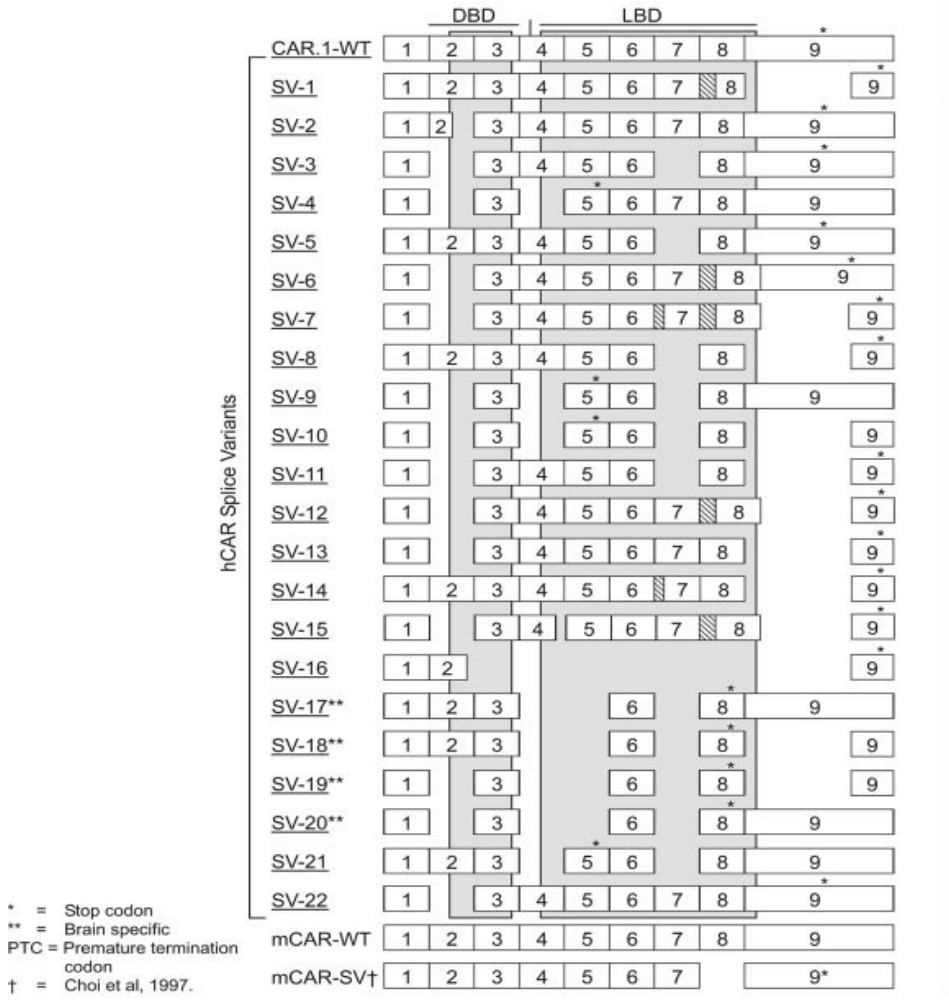


Fig. 7* Alternatively spliced variants of CAR

All the exons are numbered, shaded boxes represent the insertions, and the deletions are represented by gaps. The shaded area represents DNA and ligand binding domains.

* stop codons;

** brain-specific isoforms;

***from Lamba et al. 2004**

Human genomic diversity for any gene involves sequence variations as well as alternatively spliced mRNAs. Indeed, alternate splicing of mRNAs is considered to be one of the mechanisms generating protein diversity. According to recent studies, between 22-59% of human genes are alternatively spliced. Alternative splicing can regulate the induction of the wild-type mRNA and show gain-of-function, loss-of-function, or dominant negative activity. Polymorphic alternative splicing can cause human diseases and is a common mechanism of human variation in cytochrome P450 expression (Lamba et al. 2004).

Every NR protein molecule, including CAR, is composed of four modules: the modulator domain carrying a ligand-independent transcription activation function (AF -1); the DNA-binding domain (DBD); the hinge region (H); and the ligand-binding domain (LBD) (Nakata et al. 2006). CAR binds to DNA as a heterodimer with the retinoid X receptor (RXR). The inspection of the molecular model of hCAR-DBD/hRXRα-DBD shows that hCAR-DBD is folded into a globular shape, consisting of two a-helices perpendicular to one another (residues Glu29-Lys41, and residues Pro64-Ala74), a connector loop (residues Arg77-Ala84) that lies perpendicular to the long axis of DNA, and a long α-helix (residues Ala84-Arg97). The connector loop region is interrupted by a helical turn (residues Lys78-Asp79-Met80). Both CAR-DBD and PXR-DBD contain eight conserved Cys residues devided in two groups of four. Each group is involved in the tetrahedral coordination of a single zinc ion (di Masi et al. 2009).

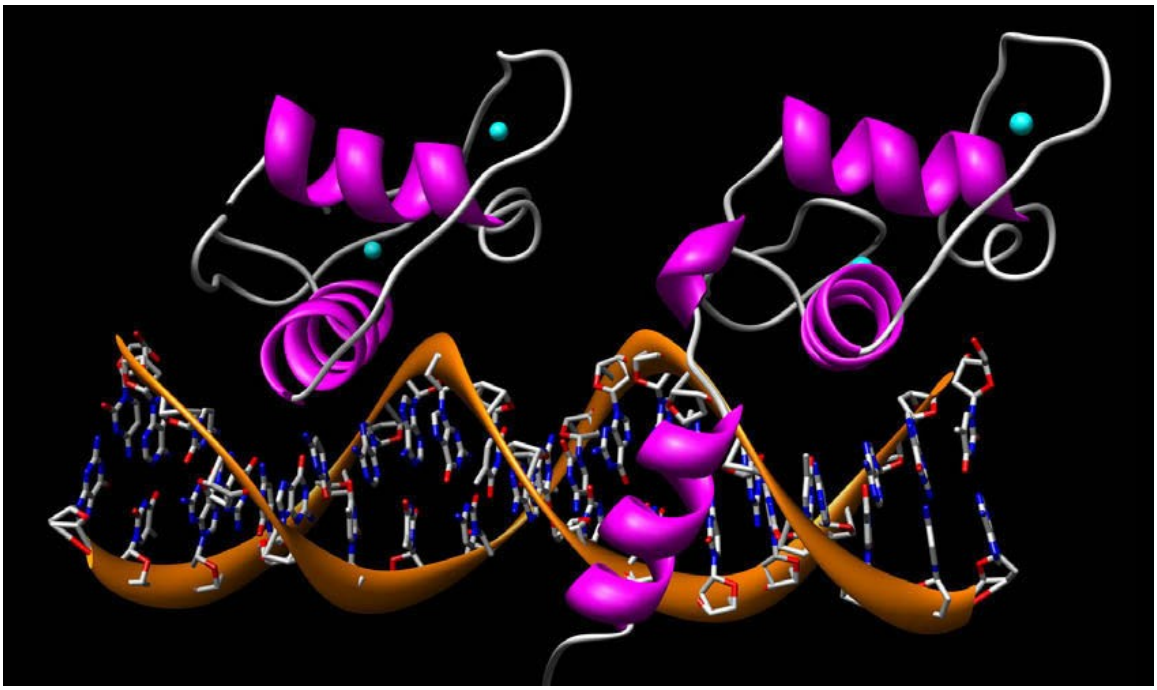


Fig. 8* Three-dimensional model of hCAR-DBD/hRXRa-DBD bound to a DNA strand

Zinc ions are represented as cyan spheres. The DBD a-helices are shown in magenta. The phosphate backbone of DNA is represented in orange. The nucleotide bases are shown in stick and colored according to the atom type.

***from di Masi et al. 2009**

hCAR-DBD and hRXRα-DBD heterodimerize in the ‘‘head-to-tail” orientation. The interaction between hCAR-DBD and hRXRα-DBD involves Tyr21, Asp79, and Asn24 of hCAR-DBD and Arg172 and Arg186 of hRXRα-DBD. In particular, hRXRa-DBD Arg172 creates a salt bridge with hCAR-DBD Asp79. Moreover, hRXRa-DBD Arg186 forms a hydrogen bond with the hCAR-DBD Asn24 carbonyl oxygen atom, and a planar stacking interaction with hCAR-DBD Tyr21. Furthermore, amino acid residues surrounding the Arg186 residue of the hRXRα-DBD, notably Asn185, Arg184 and Gln188, form hydrogen bonds to the DNA backbone and thereby support its proper position in the heterodimer interface (di Masi et al. 2009)

The CAR LBD consists of 11 α helices, two 3^{10} helices (H2 and H2´), and three small β strands that are arranged into a compact helical sandwich fold (Suino et al. 2004; di Masi et al. 2009; Xu et al. 2004). The structure indicates that the constitutive activity of CAR results from several factors including a short helix preceding the AF-2 helix, helix 12, which combines with a salt bridge between C terminus of helix 12 and helix 3 to stabilize the AF-2 helix in the active conformation. The CAR LBD is stabilized further by an extended helix 2 that makes contacts with helix 3. The CAR LBD includes a well-formed ligand-binding pocket of approximately 600 $Å^3$ (Moore et al. 2006). Despite the similar LBD fold, the CAR structure exhibits three unique features not present in other nuclear receptors. The first and most clear feature is the short and relatively rigid AF2 helix, which is the shortest among the nuclear receptors studied so far. The short length of the CAR AF2 helix allows the C-terminal free carboxylate group to interact with K205 from helix H4. Residue K205, together with other carbonyl groups (which are otherwise uncapped) in the C terminus of the AF2. These additional hydrogen bonds may further help to register the AF2 helix in the active conformation. The second unique feature of CAR is that the linker region between helices H10 and AF2 is also the shortest linker among nuclear receptors. This linker region normally adopts an extended loop configuration but in CAR adopts a two-turn α helix instead. To accommodate this linker helix, helix H10 of CAR is one turn (four residues) shorter than that of other LBDs, which allows the linker helix to pack tightly against helices H3 and H10. This unique arrangement of helix H10 and the linker helix is crucial for CAR constitutive activation, because insertion of three amino acids into this short linker disrupts CAR constitutive activity, but not TCPOBOP-induced activation. Finally, in addition to the three layers of helices found in other receptors, CAR contains

another layer consisting of two 3^{10} helices (H2 and H2´). Both helix H2 and the loop connecting helices H2 and H2´ pack closely against the N-terminal portion of H3 and help to rigidify this portion of helix (Suino et al. 2004).

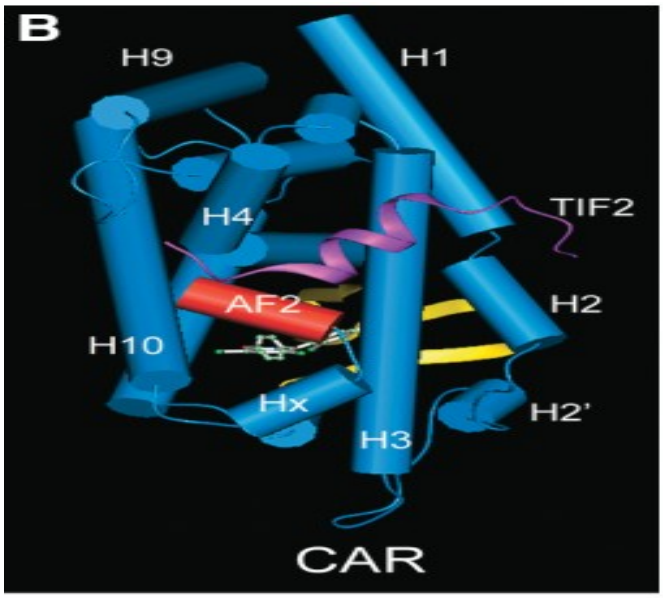


Fig. 9* Three-dimensional structure of CAR-LBD

The four layered helix sandwich of CAR LBD structure is shown in solid rendering (α helices in cylinders and β strands in yellow ribbons). TCPOBOP is shown in ball-and-stick form. Key secondary structural elements are annotated, including the linker helix Hx.

***from Suino et al. 2004**

The LBDs of the CAR and RXRα molecules create a back-to-back heterodimer along their respective H10, with SRC-1 peptides bound in each of the coactivator grooves (Xu et al. 2004). Each LBD inherits a helical sandwich fold that is conserved across the nuclear receptor family (Suino et al. 2004). In the CAR-LBD/hRXRα-LBD heterodimer, the CAR-LBD a10 helix packs parallel to the hRXRα-LBD a10 helix and contacts the hRXRα-LBD aAF helix. Moreover, the N-terminal end of hCAR-LBD a7 helix is near hRXRα-LBD aAF helix. However, the relatively short CAR-LBD aAF helix restricts the possibility of interaction with hRXRα-LBD (di Masi et al. 2009).

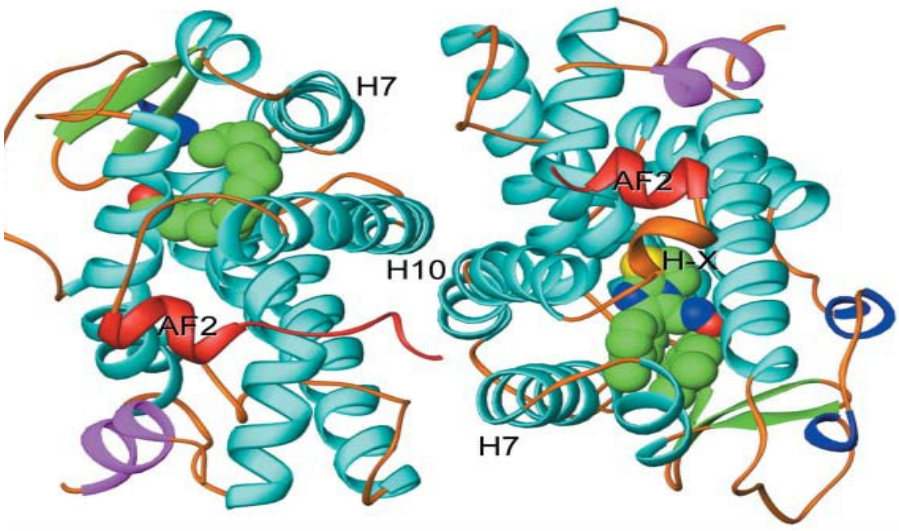


Fig. 10* Ribbon diagram of the human CAR/RXR heterodimer complex

The SRC-1 peptides are in magenta. The 3^{10} helixes of CAR and RXR are colored blue. The AF2 helices are colored in red. Helix X (H-X) is colored in gold. H7 and H10 are annotated. CITCO in CAR and the endogenous fatty acid in RXR are shown in space-filling representation colored by atom type: oxygen is red, nitrogen is blue, sulfur is yellow, and carbon is green.

***from Xu et al. 2004**

The mechanism of CAR activation is novel. In contrast to most orphan nuclear receptors that are situated in the nucleus, CAR is compartmentalized in the cytoplasm in a similar way to classic steroid hormone nuclear receptors such as GR. Retention in the cytoplasm prevents chronic activation of CAR target genes and allows controlled regulation of the receptor's activity. A phosphorylation cascade is involved in regulating CAR nuclear translocation, yet elucidation of the CAR activation mechanism has proved difficult because of the fact that CAR accumulates spontaneously in the nucleus of transformed cell lines regardless of activation state (Swales et al. 2004).

CAR has both direct and indirect mechanisms of activation based on either conventional agonist binding or a still poorly characterized pathway of induced nuclear translocation. In the second case, the constitutive transactivation action of CAR results in initiation of expression of appropriate target genes. There are no known endogenous agonists that directly activate CAR in physiologic pathways (Moore et al. 2006).

Indeed, CAR is characterized by high constitutive activity in cell-based transfection assays. In computer-generated models, CAR contains the conserved activation function 2 (AF2) domain and a standard ligand binding (LB) cavity lined with hydrophobic residues,

that support the ability of this receptor to bind specific ligands such as androstanol, TCPOBOP, and CITCO (Swales et al. 2004). 6-(4-chlorophenyl) imidazo[2,1-*b*] [1,3]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime, best known as CITCO is a potent and highly selective human CAR agonist (Maglich et al. 2003). Following this, HMG-CoA reductase inhibitors (cerivastatin, simvastatin, fluvastatin, and atorvastatin) increased the hCAR-mediated transcriptional activation of phenobarbital-responsive enhancer module reporter gene by up to 3-fold (Kobayashi et al. 2005)**.** However, the first CAR ligands identified were the endogenous androgen metabolites, androstanol and androstenol, which are inverse agonists, able to block the constitutive activity of CAR, but this requires micromolar concentrations that are way above those reached in vivo (Moore et al. 2006).

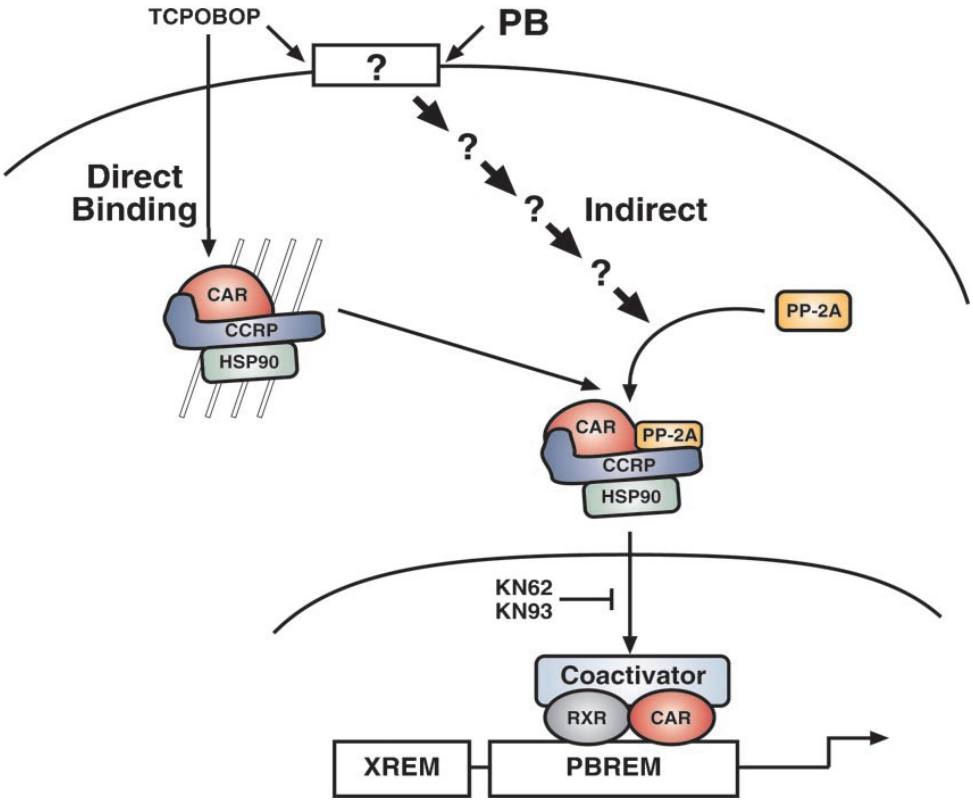


Fig. 11* Mechanism of CAR Activation

CAR translocation can be initiated by either direct ligand binding to the receptor, or indirectly, via a partially elucidated signal transduction pathway. CAR exists in a complex with Hsp90, retained in the cytoplasm by the cochaperone CCRP. Indirect activators or the direct binding of ligands to CAR subsequently recruit PP2A to the complex. Once in the nucleus, more activation steps involving calmodulin-dependent kinase and recruitment of coactivators occur before DNA binding and transcriptional activation of target genes.

***from Swales et al. 2004**

CAR is situated in the cytosol in a multi-protein complex which includes a recently identified protein called CAR cytoplasmic retention protein (CCRP) and the heat shock chaperone Hsp90. CCRP overexpression in human hepatoma cells, promotes CAR accumulation in the cytosol; therefore, upon TCPOBOP stimulation, a more intensed nuclear translocation of the xenoreceptor occurs (di Masi et al. 2009). Translocation of CAR to the nucleus is followed by heterodimerization with the retinoid X receptor (RXR) and binding to the PBREM. Transcriptional activation occurs upon CAR binding to the PBREM which is composed of two nuclear receptor DR4 sites (NR1 and NR2) (Timsit et al. 2007). It was first believed that nuclear translocation led to the activation of CAR. However, the $Ca2^{+}$/ calmodulin-dependent kinase inhibitor KN-62 suppressed induction of CYP2B10 mRNA as well as the activation of an NR1-reporter gene by both PB and TCPOBOP in mouse primary hepatocytes, but it did not prevent PB-induced accumulation of CAR in the nuclei, suggesting that CAR may undergo a distinct activation process in the nucleus (Swales et al. 2004).

An expanding number of coactivators and corepressors that interact with CAR have been identified, such as SRC-1, Xenopus SRC-3, glucocorticoid receptor-interacting protein 1, and peroxisome proliferator activated receptor-γ coactivator 1α, which may simultaneously regulate CAR via different regions of the receptor (Swales et al. 2004). The interaction of CAR with the p160 coactivator glucocorticoid receptor interacting protein 1 (GRIP1) has been investigated *in vitro* and *in vivo*. Remarkably, CAR and GRIP1, combined together, synergistically transactivated the enhancer about 150- fold, which is about equal to activation by PB treatment. In PB-treated mice, expression of exogenous CAR alone had low effect, expression of GRIP1 increased transactivation about 2-fold, and with CAR and GRIP together, a 4-fold activation was observed. In untreated mice, induction of GRIP resulted in nuclear translocation of green fluorescent protein-CAR. These results strongly suggest that a p160 coactivator acts in CAR-mediated transactivation *in vivo* in response to PB treatment and that the synergistic activation of CAR by GRIP in untreated animals results from both nuclear translocation and activation of CAR (G. Min et al. 2002). Equally important, ligand-independent activation of CAR is accomplished in cooperation with the peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). PGC-1β (a PGC-1α homologue) also activates CAR to a less extent than PGC-1α. Co-expression of the ligand-binding domain of a hetero-dimerization

partner, retinoid X receptor α, enhances the PGC-1α-mediated activation of CAR, although it has a low effect on the basal activity of CAR in the absence of PGC-1α. Both the N-terminal region, with the LXXLL motif, and the C-terminal region, with a serine/arginine-rich domain (RS domain), in PGC-1α are required for full activation of CAR. Pull-down experiments using recombinant proteins showed that CAR directly interacted with both the L*XX*LL motif and the RS domain. Furthermore, it was demonstrated that the RS domain of PGC-1α is needed for CAR localization at nuclear speckles. These results indicate that PGC-1α mediates the ligand-independent activation of CAR by means of subnuclear targeting through the RS domain of PGC-1α (Shiraki et al. 2003).

3.2 Function of CAR in energy homeostasis

CAR was originally defined as xenobiotic receptor, regulating the expression of drug-metabolizing enzymes and transporters as adaptive responses to prevent the accumulation of toxic chemicals in the body (Wada et al. 2009). CAR coordinates the regulation of various hepatic genes resulting, mainly in metabolic detoxification by CYPs and transferases within the hepatocyte, followed by the net clearance of xenobiotics, by transporters such as OATP2 ,from the blood (di Masi et al. 2009).

3.2.1 Role of CAR in gluconeogenesis and lipid metabolism

Activation of CAR might suppress lipid metabolism and lower serum triglyceride levels by reducing levels of SREBP-1, a major regulator of lipid metabolism. The inhibitory effects of CAR on lipid metabolism can be also attributed to induction of Insig-1, a protein with antilipogenic properties (Wada et al. 2009). Insig-1 and Insig-2 are proteins of the endoplasmic reticulum (ER) membrane and play an important role in the control of triglyceride and cholesterol biosynthesis. The two isoforms bind in a sterol-dependent pathway to another ER membrane protein, sterol regulatory element binding protein (SREBP) named sterol cleavage-activating protein (SCAP) a transport protein needed for escort and subsequent activation of SREBP transcription factors. When Insig proteins are

induced by sterols, insulin or other stimuli, they retain the SCAP-SREBP complex in the ER membrane, thereby preventing SREBP-dependent target gene expression. SREBPs are a group of basic helix-loop-helix transcription factors, which express an array of genes involved in the synthesis of cholesterol and triglycerides. Whereas, SREBP-2 is primarily involved in cholesterol biosynthesis, SREBP-1a and -1c mainly activate genes involved in fatty acid and triglyceride synthesis (Roth et al. 2008).

CAR might also regulate energy metabolism by interacting with the peroxisome proliferator-activated receptor α (PPARα) and PGC-1α (PPARγ coactivator-1α), both of which are key regulators of adaptive responses to starvation (Gao et al. 2009). PGC-1a enhances CAR activity in a ligand-independent manner and, also, potentiates the transcriptional activity of CAR in the presence of CAR agonists. An evolutionarily conserved HNF4α-response element (HNF4-RE) situated in the CAR-proximal promoter region has been identified and might explain the coordinated induction of PGC-1a and CAR during fasting (Wada et al. 2009). HNF-4α is a positive regulator of gluconeogenesis. It was suggested that CAR suppresses gluconeogenic enzyme gene expression through HNF-4α inhibition, achieved by the competition of CAR with HNF-4α for binding to the DR1 motif in the promoter region of gluconeogenic enzyme genes. Equally important, FOXO1 is also a major positive regulator of gluconeogenesis. It has been reported that CAR can physically bind to and inhibit the activity of FOXO1 by preventing FOXO1 from binding to the insulin response sequence in gluconeogenic enzyme gene promoters (Gao et al. 2009). FOXO1 is an activator of gluconeogenic genes, such as PEPCK1, G6P, and insulin-like growth factor-binding protein 1. These gluconeogenic genes consist of an insulin response sequence (IRS) to which FOXO1 can bind directly and activate in the absence of insulin (Kodama et al. 2004).

Moreover, peroxisome proliferator-activated receptor a (PPARα) agonists (e.g. fibrates) are a frequent treatment of diabetic dyslipidemia/primary hypertriglyceridemia. One of the main effects of PPARα activation by fibrates is decreased hypertriglyceridemia due to enhanced free fatty acid b-oxidation in the liver. The nuclear receptor PPARα stimulates gene transcription by binding to peroxisome proliferators response elements (PPREs) in the promoter of target genes (Maglich et al. 2009). CAR has been reported to interact with fatty acid metabolism by binding to DNA elements overlapping with the PPARα-binding site in the promoter region of the 3-hydroxyacyl-CoA dehydrogenase, an important enzyme of peroxisomal fatty acid b-oxidation (Wada et al. 2009).

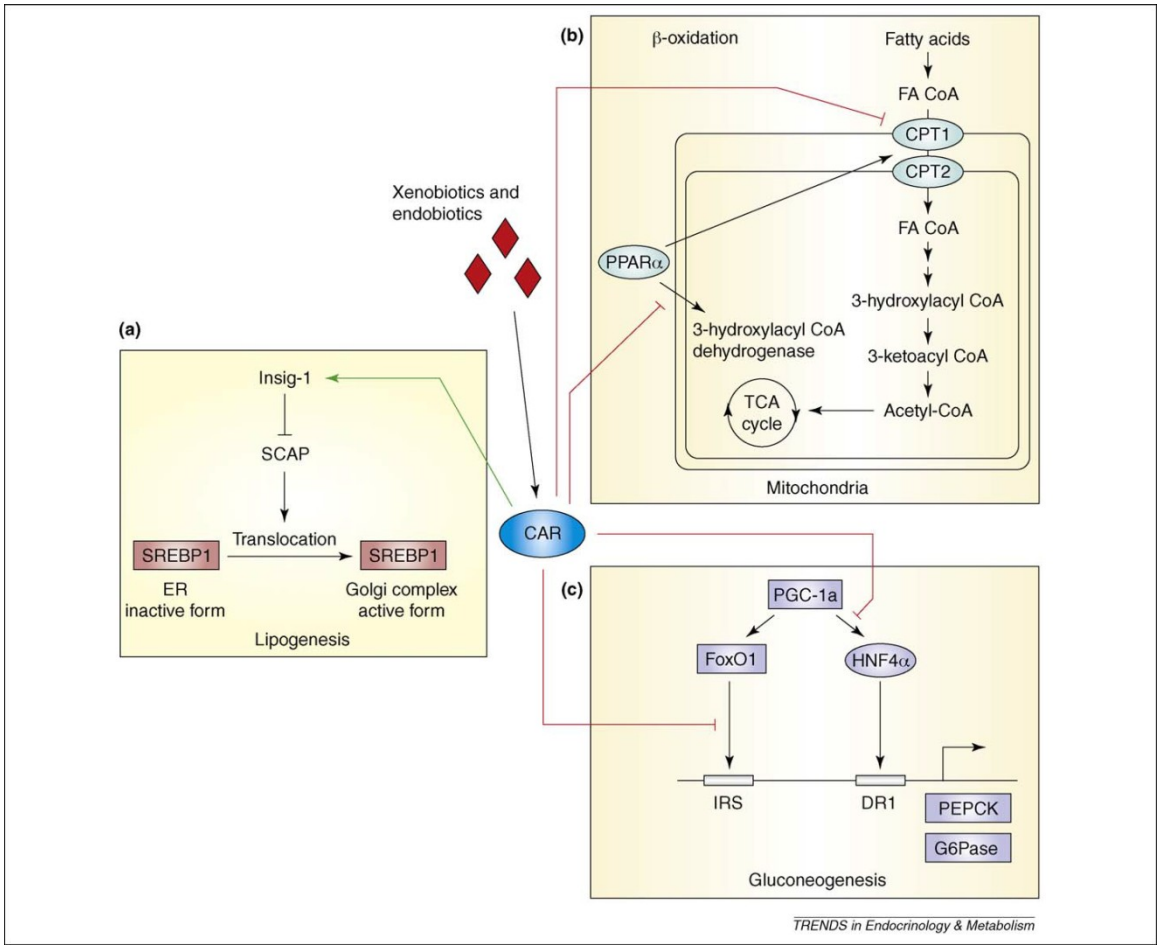


Fig. 12* Representation of the role of CAR in gluconeogenesis and lipid homeostasis

CAR functions as a negative regulator of lipid metabolism, fatty acid b-oxidation, and gluconeogenesis. Arrows and stop bars refer to positive regulation, or activation, and negative regulation, or repression, respectively.
(a) CAR suppresses lipogenesis by inducing Insig-1, a protein that has a role in SREBP-mediated regulation of lipogenic genes. Insig proteins bind and trap SCAP, retaining it in the ER and preventing it from escorting SREBPs to the site of proteolytic activation in the Golgi complex. SREBPs are cleaved by two proteases in the Golgi complex, and the bHLH-Zip domain of SREBPs transfers from the membrane to the nucleus to bind the sterol response elements in the promoter region of target genes.
(b) CAR inhibits fatty acid b-oxidation. CAR competes with PPARa for its binding site in the 3-hydroxyacyl-CoA dehydrogenase gene promoter. Activation of CAR also reduces the expression of CPT1, a rate-limiting enzyme of b-oxidation.
(c) CAR inhibits gluconeogenesis. CAR binds to FOXO1, resulting in suppression of FOXO1 target gene expression. CAR also competes with HNF4a for its binding site (DR1) and competes with HNF4a for NR co-activators.

***from Wada et al. 2009)**

3.2.2 Role of CAR in steroid and thyroid hormone homeostasis

The first endogenous modifiers of CAR activity discovered were the steroids androstanol and androstenol, which suppress the activity of the receptor by dissociating the interaction between CAR and SRC-1. Similarly, progesterone and testosterone inhibit the constitutive activity of CAR. In contrast, pharmacological levels of E2 and estrone as well as the progesterone metabolite pregnane-3,20-dione can induce rodent and human CAR, respectively (di Masi et al. 2009).

It has been suggested that CAR responds to nutrient stress, evident by its induction during long-term fasting. One possible mechanism by which CAR affects fasting responses is via its regulation of thyroid hormone (TH) metabolism, through regulation of phase II TH-metabolizing enzymes, such as UGT1a1, SULT1a1, SULTn and SULT2a1 (T. Wada et al. 2009).The phase II drug-metabolizing enzymes uridine 5′-diphosphate glucuronosyltransferase (UGT) and sulfotransferase (SULT) regulate the glucuronidation and sulfation of TH. Expression of these enzymes by the widely used antiepileptic drug phenobarbital (PB) and other xenobiotics enhances TH metabolism and reduces serum TH levels in both animals and humans. Activation of CAR by PB or the potent and more effective agonist ligand 1, 4-bis- [2-(3, 5,-dichloropyridyloxy)] benzene (TCPOBOP) leads to a decrease in the serum TH levels. This is accompanied by an elevation in serum TSH levels and a subsequent increase in thyroid follicular cell proliferation. This effect of CAR depends on its ability to initiate the expression of both UGT and SULT enzymes that are involved in TH metabolism and excretion (Qatanani et al. 2005).

3.2.3 Role of CAR in bilirubin metabolism and heme biosynthesis

Bilirubin is an oxidative end product of heme catabolism. A significant amount of bilirubin is produced each day (250–400 mg in adult humans), mainly from breakdown of hemoglobin. It is very hydrophobic and in chronic jaundice it concentrates in the CNS, developing neurotoxicity that can result in potentially fatal encephalopathy (Huang et al. 2002). Glucuronidation by uridine diphosphate glucuronyl transferase (UGT1a1) is the principal detoxification pathway of bilirubin (di Masi et al. 2009). Bilirubin is glucuronidated in the endoplasmic reticulum and secreted through the bile–canalicular

membrane of the hepatocyte by an active transporter, known as MRP2 (multidrug resistance-associated protein 2) (Huang et al. 2002). For a long time it has been known that PB could decrease increased bilirubin levels. Nowadays, this appears to be due to the ability of CAR to promote the bilirubin excretion by inducing the biliary transporters UGT1A1, MRP2, OATP2, and GST A1 (di Masi et al. 2009; Huang et al. 2002).

The induction of 5-aminolevulinic acid synthase 1 by PB seems to indicate that CAR may also regulate the induction of heme biosynthesis by elevating CYP levels. However, induction studies of PB and TCPOBOP in CAR-null mice show that the regulation of 5-aminolevulinic acid synthase 1 is CAR independent (di Masi et al. 2009).

3.2.4 Role of CAR in bile acid homeostasis

Bile acids, the amphipathic end products of cholesterol metabolism, are crucial for the absorption of dietary fats and fat-soluble vitamins, as well as regulation of bile flow and biliary lipid secretion that control the excretion of conjugated drugs and endogenous waste products. Bile acid production also represents a major way for the elimination of excess cholesterol. A role for CAR in protection against bile acid toxicity was confirmed by a significant reduction of serum bile acid and bilirubin concentrations, with an increase in the expression of the hepatic genes involved in bile acid and/or bilirubin metabolism and excretion (CYP2B, CYP3A, UGT1A, MRP2, MRP3, and glutathione *S*-transferase a), following pretreatment with Phenobarbital or TCPOBOP (Guo et al. 2003). Two nuclear hormone receptors, Farnesoid X receptor (FXR) and PXR, have been identified as important regulators of bile acid metabolism in the liver. FXR can be induced by many bile acids and mediates the expression of metabolizing enzymes and transporters in the bile acid homeostasis pathway. The numerous target genes of FXR involve cholesterol 7a-hydroxylase (CYP7A), sterol 12-a hydroxylase (CYP8B1), the membrane transporters bile salt export pump (ABCB11), Na^+-dependent taurocholate cotransporting polypeptide, multidrug resistance-associated protein 2 (MRP2) (ABCC2), and the sulfotransferase STD. PXR also acts in bile acid homeostasis. Activation of PXR by pregnane-16a-carbonitrile induces CYP3A, STD, and Na^+-dependent organic anion transporter 2 (OATP2), which are crucial for lithocholic acid (LCA) detoxification and transport (Zhang et al. 2004).

3.3 Regulation of CYP450s by CAR

Cytochromes P450 (CYPs) are a superfamily of proteins, members of which catalyze the metabolism of a diverse array of endogenous and exogenous chemicals. CYP-mediated detoxification is a major defense mechanism whereby organisms protect themselves from the potentially harmful effects of foreign hydrophobic chemicals to which they are exposed. The xenobiotics are transformed to more hydrophilic compounds, which are more easily excreted (Smirlis et al. 2001). The primary stage in the detoxification of xenobiotics is usually carried out by cytochrome P450s (CYPs), in particular by members of the CYP1, CYP2, CYP3 and CYP4 families (Muangmoonchai et al. 2001).

The PB-responsive enhancer module (PBREM), a flexible enhancer capable of responding to various PB-type inducers, regulates PB induction of the CYP2B genes in mouse, rat, and human cells. PBREM consists of two DR-4 nuclear receptor-binding motifs, NR1 and NR2. Acting as a retinoid X receptor (RXR) heterodimer, the liver-enriched constitutively active receptor (CAR) increases its binding to NR1 in PB-treated mice (Kawamoto et al. 1999). CAR initiates the expression of a reporter gene attached to the phenobarbital response element (PBRE) of the cytochrome P450 2B1 (CYP2B1) gene in response to the barbiturate phenobarbital. Steroid co-activator 1 (SRC-1) increases both constitutive and xenobiotic-induced CAR-mediated transactivation via the CYP2B1 PBRE in transfected primary hepatocytes. By binding to the proximal promoter of CYP2B1, the transcription factor Sp1 enhances both basal transcription and xenobiotic-induced expression via the PBRE. Thus induction of CYP2B1 expression by xenobiotics is regulated by the nuclear receptor CAR and, for optimal expression, requires SRC-1 and Sp1 (Muangmoonchai et al. 2001). In addition to that, the results of the investigation by T. Sueyoshi et. al. show unequivocally that CAR, through its binding to the NR1 site of PBREM, regulates induction of the endogenous CYP2B6 gene in response to PB and TCPOBOP. Furthermore, K. Inoue et al. have now identified the cohesin protein SMC1 as a CAR-binding protein and characterized it as a negative regulator of okadaic acid response element (OARE) activity. Treatment with SMC1 small interfering RNA enhanced the synergistic up-regulation of CYP2B6 expression 20-fold in HepG2 cells. Both DNA affinity and chromatin immunoprecipitation assays prove that OA treatment dissociates SMC1 from the CYP2B6 promoter, reciprocating the indirect binding of CAR to OARE.

These results are consistent with the conclusion that SMC1 binding suppresses OARE activity and its dissociation allows the recruitment of CAR to the OARE, synergizing PBREM activity and the expression of the CYP2B6 gene (K. Inoue et al. 2006).

Moreover, CAR is capable of trans-activating expression of the CYP3A4 gene, which is the prevailing P450 expressed in adult human liver. CAR responsiveness is proved to be primarily mediated by two high-affinity binding motifs situated within the CYP3A4 gene 5´-flanking region, about 7720 and 150 bases upstream of the transcription initiation site. Importantly, the human CAR response elements also mediate trans-activation of CYP3A4 by the human pregnane X receptor, suggesting that synergism between these receptors is likely to be an important determinant of CYP3A4 expression (Goodwin et al. 2002).

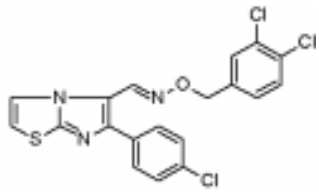
Lastly, The CYP2C subfamily is an important class of enzymes composed of four isoforms (CYP2C9, CYP2C19, CYP2C8, and CYP2C18) that are supposed to metabolize approximately 20% of known drugs. The CYP2C9 isoform is the most clinically important member of this subfamily. CAR transfection can up-regulate CYP2C9 mRNA content. Upon cloning and analyzing reporter assays with a longer 3-kb segment of the CYP2C9 promoter, there was a significant increase in reporter activity with the cotransfection of hCAR. Further investigation revealed that the presence of the CAR-RE (NR1) portion of the 2898 module was sufficient to confer hCAR mediated activation to the basal CYP2C9 promoter in both HepG2 cells and primary human hepatocytes. It was also found that hCAR expression enhanced reporter activity of the CAR-RE response element in a concentration-dependent manner. This increase is consistent with a specific interaction between the receptor and the CAR-RE. These findings indicate that the CAR-RE is involved in hCAR mediated activation of CYP2C9 transcription (Ferguson et al. 2002).

4.The aim

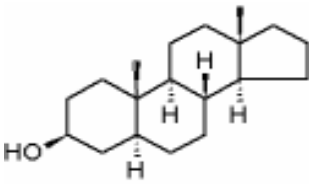
The aim of the diploma thesis is to summarize current knowledge about nuclear receptors from the last years and to search new leads about a specific orphan nuclear receptor called constitutive androstane receptor (CAR). For this purpose, we investigate interactions of 11 selected newly synthesized compounds with constitutive androstane receptor (CAR) employing in vitro gene reporter cellular assays.

5. Experimental Part

Schematic representation of the tested compounds



CITCO, 6-(4-Chlorophenyl)imidazo[2,1-b][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime

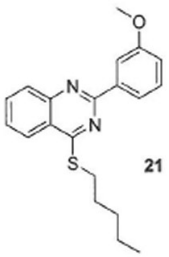


3β - Hydroxy- 5α – androstane

Androstanol

Tested compounds

Compound	Molecular Weight
21	338,47
22	352,49
23	308,40
24	358,46
25	286,35
26	254,31
27	270,37
28	284,33
29	314,43
30	280,34
CHP3	252,27
CHP12	251.28



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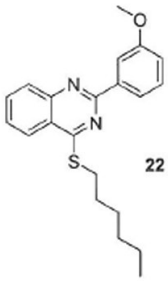

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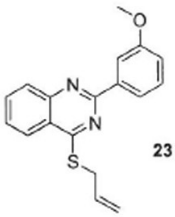

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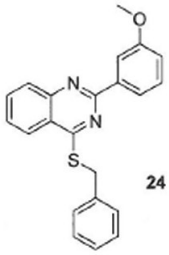

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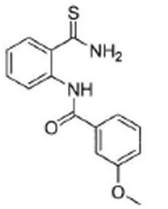

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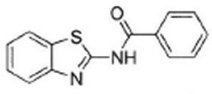

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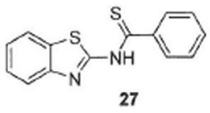

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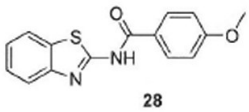

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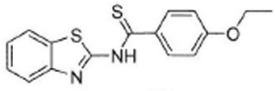

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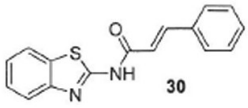

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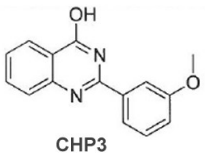
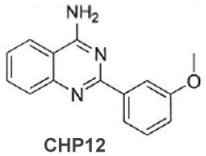


CHP3



CHP12

The compounds were kindly provided by Dr. Špulák

5.1 Materials

Cell line

The human **MZ-Hep-1** hepatocarcinoma cell line (kindly donated by Dr. Ramiro Jover, Hospital La Fe, Valencia, Spain) was maintained in antibiotic-free DMEM supplemented with 10% FBS and 1 mM sodium pyruvate. Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria). CITCO was purchased from BIOMOL (Plymouth Meeting, PA). Other chemicals and cell culture media were purchased from Sigma–Aldrich (St. Louis, MO). The final concentration of DMSO in the culture media was 0.1% (v/v) in all experiments.

DNA constructs

The human CAR expression plasmid **pCR3-hCAR** was kindly provided by Dr. M. Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC).
p(ER6)$_3$-luc plasmid was synthesized using complementary pairs of oligonucleotides containing three tandem copies of ER6 of the *CYP3A4* promoter (5-CTAGCATATGAACTCAAAGGAGGTCAGTGGATGGAATGAACTCAAAGGAGGTCAGTGGATGGAATGAACTCAAAGGAGGTCAGTGA-´3). Oligonucleotides were annealed and cloned into the NheI- and BglII-digested sites of the **pGL4.23** vector containing a minimal promoter (Promega).

Transient Transfection and Luciferase Gene Reporter Assays

Cells were maintained in phenol red-free medium (200 µl) supplemented with 10% charcoal/dextran-stripped FBS. Luminescence activity was determined with a **Genios Plus luminometer** (Tecan, Gro¨dig, Austria) in cell lysate usinga commercially available luciferase detection system (Dual Luciferase Reporter Assay Kit; Promega).

5.2. Methods

MTS assay

The first assay that was used is a colorimetric method, which is applied to determine the proliferation, viability and activation of cells used in further assays. In brief, we search to find if the cells are healthy enough to continue our investigation. MTS assay is composed of a novel tetrazolium compound, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; better known as MTS and an electron coupling reagent termed PMS (Phenazine Methosulfate). The MTS is bioreduced by the healthy cells into a formazan product that is soluble in tissue culture medium.

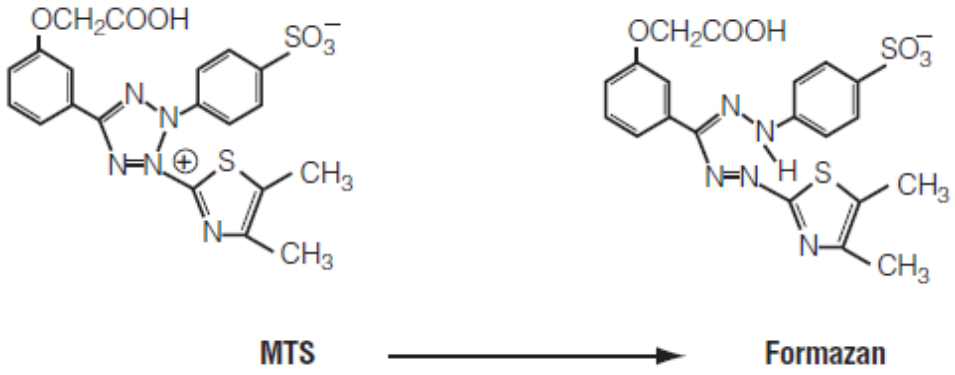


Fig. 13* Schematic representation of MTS tetrazolium salt and the conversion to its formazan product

The conversion of MTS into aqueous, soluble formazan is accomplished by dehydrogenase enzymes of metabolically active cells.

***from CellTiter 96 Non-Radioactive Cell Proliferation Assay, Technical Bulletin**

A high number of MZ-Hep1 cells in phenol red-free medium, supplemented with 10% charcoal/dextran-stripped FBS, were treated with the tested compounds (85μL) into a 96-well plate. Simultaneously, the cells were treated with vehicle (DMSO; 0.1%, v/v). After 24 hours of the transfection, we emptied the cells and added 100μl of fresh culture media. Then, we pipetted 20μl of the MTS/PMS One solution (20:1 ratio) into each well of the 96 well plate containing the 100μl of cells in fresh culture media. Then, we incubated the plate for one hour at 37ºC in a 5% CO_2, humidified atmosphere. Finally, we recorded the absorbance at 540nm of the bioreduced formazan product (blue product) with the use of a TECAN plate reader provided by Schoeller Instruments LLC.

Gene reporter assay

Gene reporter method is based on two assays: (i) the Mammalian two-hybrid assay and, (ii) the Dual-luciferase reporter assay, which makes quantitation of luciferase reporter genes a rapid and easy process.

(i) The two-hybrid system is an extremely powerful way to detect protein:protein interactions in vivo. The system works as follows: the DNA-binding domain and the transcriptional activation domain, produced by two separate plasmids (pACT and pBIND), become closely associated when one protein (CAR) fused to a DNA-binding domain interacts with a second protein (pRL-TK control plasmid) fused to a transcriptional activation domain. This way the interaction between the two proteins results in transcription of the firefly luciferase reporter gene.

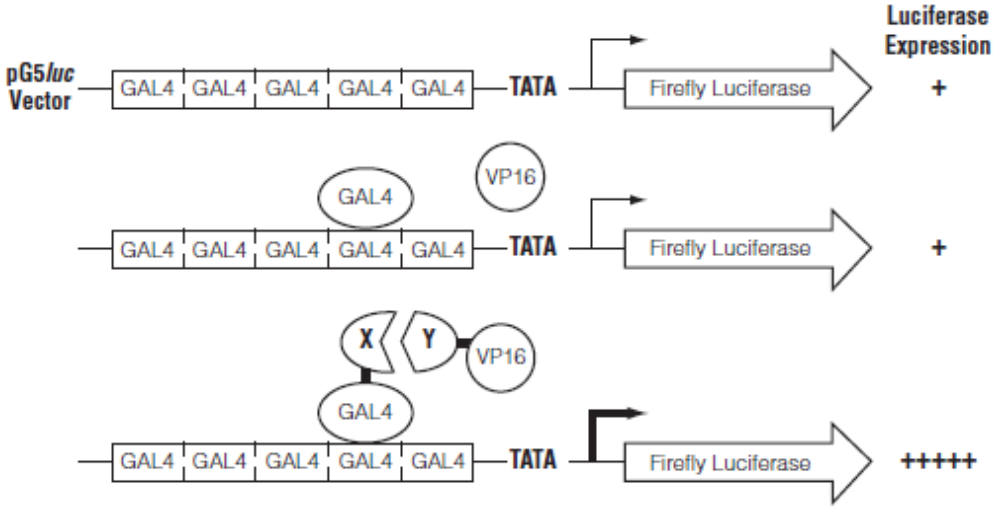


Fig. 14* Schematic representation of the Mammalian Two-Hybrid system

Instead of the pG5luc, we used the pGL4,23 vector situated upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene. Interaction between the two test proteins, expressed as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression.

***from CheckMate Mammalian Two-Hybrid systém, Technical Bulletin**

The pBIND Vector contains a yeast known as GAL4 DNA-binding domain. The pACT Vector contains the herpes simplex virus VP16 activation domain. Additionally, the pBIND Vector expresses the Renilla reniformis luciferase under the control of the SV40 promoter, which allows the user to normalize for differences in transfection efficiency. Two genes encoding two potentially interactive proteins of interest are cloned into the pBIND and pACT Vectors to generate fusion proteins with the DNA-binding domain of GAL4 and the activation domain of VP16, respectively. The pACT Vector is a high-copy plasmid in which the human cytomegalovirus (CMV) immediate early promoter drives expression of the herpes virus VP16 activation domain. On the othe hand, the pBIND Vector is a high-copy plasmid in which the CMV immediate early promoter drives expression of a portion of the yeast GAL4 gene containing a DNA-binding domain.

Eventually, the pGAL4 and pVP16 fusion constructs are transfected along with the pGL4,23 Vector into mammalian cells. 24 hours after the transfection, the cells are lysed, and the amount of Renilla luciferase and firefly luciferase are quantitated using the Dual-luciferase Reporter Assay systém.

(ii) In the Dual-Luciferase Reporter Assay, the activities of firefly (*Photinus pyralis*) and *Renilla* (*Renilla reniformis*) luciferases are measured sequentially from a single sample. The term "dual-reporter" refers to the simultaneous expression and measurement of two individual reporter enzymes within a single system. The firefly luciferase reporter is measured first by adding Luciferase Assay Reagent II (LAR II) to generate a stabilized luminescent signal. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is simultaneously initiated by adding Stop & Glo Reagent to the same tube. The Stop & Glo Reagent also produces a stabilized signal from the *Renilla* luciferase, which decays slowly over the course of the measurement.

Firefly luciferase is a 61kDa monomeric protein that does not require posttranslational processing for enzymatic activity. Thus, it functions as a genetic reporter immediately upon translation. Photon emission is achieved through oxidation of beetle luciferin in a reaction that requires ATP, Mg2+ and O2. The luminescence from the firefly luciferase reaction may be quenched while simultaneously activating the luminescent reaction of *Renilla* luciferase. *Renilla* luciferase, a 36kDa monomeric protein, is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis*. However, like firefly luciferase, post-translational modification is not required for its activity, and the enzyme may function as a genetic reporter immediately following translation. Luminescence was determined with a Genios Plus luminometer

The transient transfection reporter assays were carried out using Lipofectamine2000 reagent (Invitrogen) according to the manufacturer's instruction. MZ-Hep1 cells (2×10^5) were seeded into 48-well plates and transfected with a luciferase reporter construct *p(ER6)$_3$-luc* (150 ng/well), the expression plasmid *pCR3-hCAR* encoding CAR (50 ng/well), and *Renilla reniformis* luciferase transfection control plasmid *pRL-TK* (30ng/well) 24 h later .

Separately, to prepare the media we added in a 24-well plate, 1mL of fresh culture media plus 1μL of each stock solution (20mM), Clotrimazol, CITCO and DMSO. This way we prepared a 20μM stock solution in 1mL of media. Later on, we incubated the plate in 37^{o}C for about 1 hour. After 5 minutes, we added 200μL of media of each stock solution

into the pre-prepared cells, that stayed for 2 to 3 days after the transfection to be lysed. After 24 hours, we added 100µL of luciferin reagent into each well and measured the luminescence. Luminescence was quantified with the use of a Genios Plus luminometer.

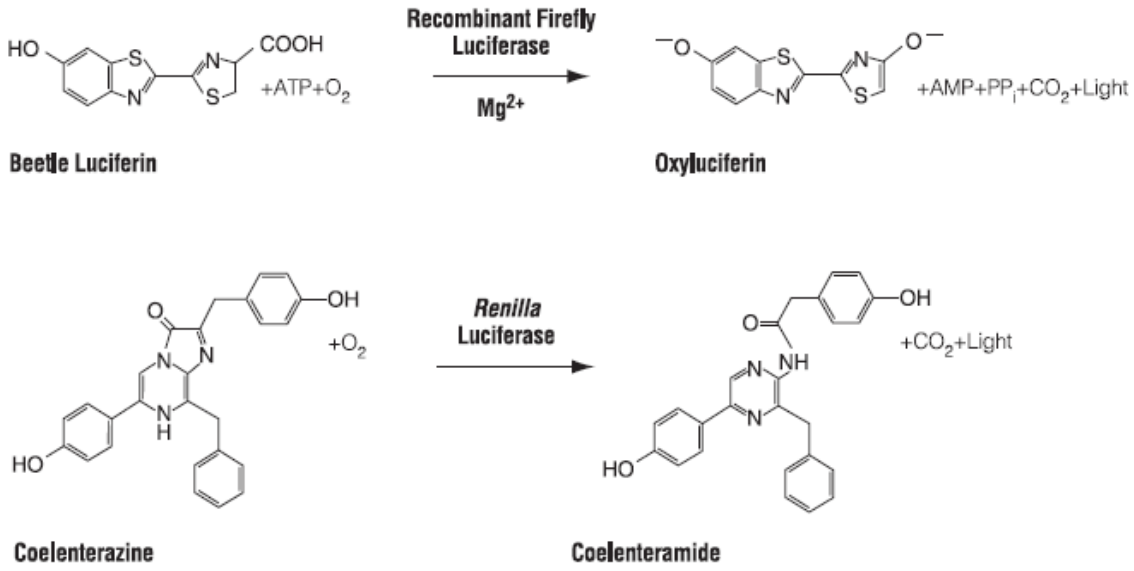


Fig. 14* Schematic representation of the bioluminescent reactions catalyzed by firefly and renilla luciferases

The luminescent reaction catalyzed by *Renilla* luciferase utilizes O2 and coelenterate-luciferin.

***from Dual-Luciferase Reporter Assay systém, Technical Bulletin**

6. Results

First, we evaluated toxicity of the tested compounds using MTS assay.

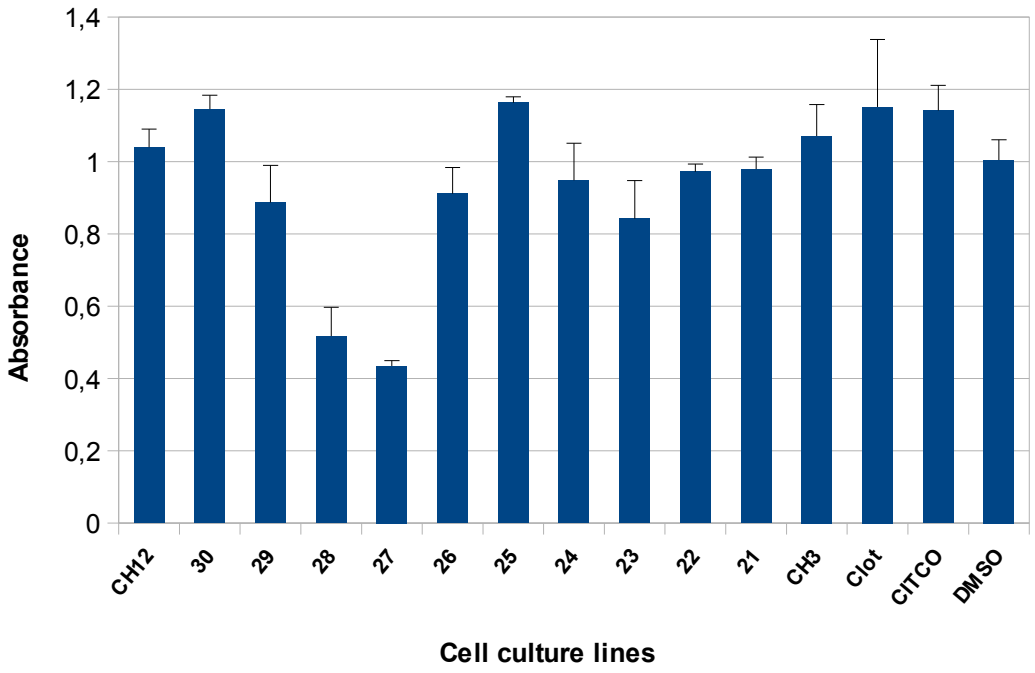


Fig. 15 ***MTS/Formazan cytotoxicity assay***

Each cell culture line shows the average absorbance of 3 different wells tested by the same compound. Looking at the data, we observe that the absorbance of the majority of the cells range from 0,8 to 1,2 which indicates that the cells are generally non-toxic and there is no fear to use these cells. The only exception are cells tested with compounds 28 and 27 that range between 0,4 and 0,6 and show that these cells are toxic. They might have gone through some kind of contamination or have been infected during the transfection of the tested compounds. The absorbance was recorded at 540 nm with the use of a TECAN plate reader.

MZ-Hep1 cells were transfected with a luciferase reporter construct p(ER6)$_3$-luc (150 ng/well), the expression plasmid encoding CAR (50 ng/well), and *Renilla reniformis* luciferase transfection control plasmid (pRL-TK, 30ng/well). 24 h later, cells were maintained in phenol red-free medium (200 µl) supplemented with 10% charcoal/dextran-stripped FBS with tested compounds for 24 h at the concentration of 20µM.

Luminescence activity was determined with a Genios Plus luminometer in cell lysate using a commercially available luciferase detection system (Dual Luciferase Reporter Assay Kit; Promega). Activity of Firefly luciferase was normalized to Renilla luciferase for all samples. Data represent the mean of three independent experiments and are reported as the fold activation of normalized luciferase aktivity relative to the solvent (0.1% DMSO) control.

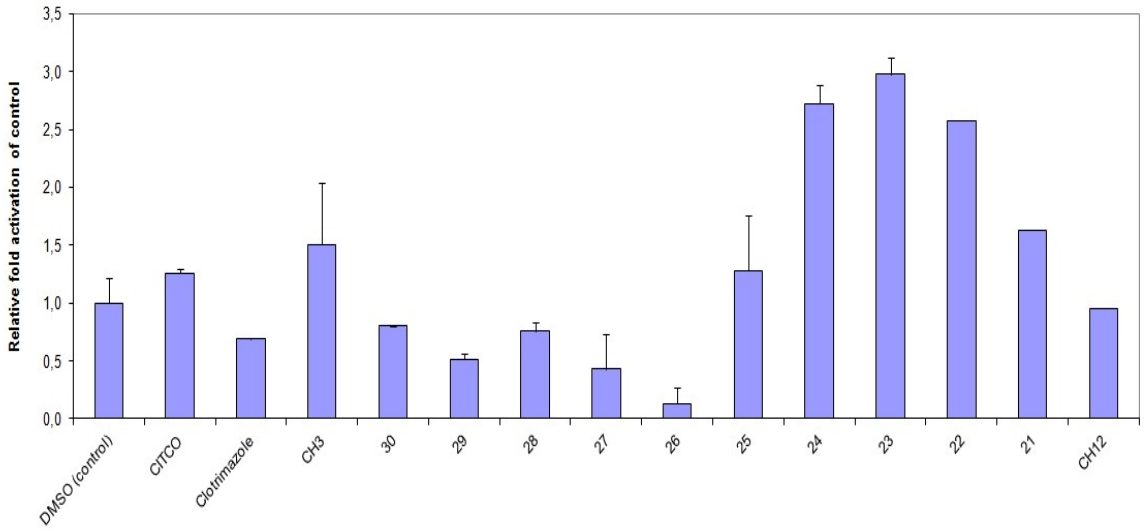


Fig. 16 ***Activation of p(ER6)$_3$-luc reporter construct by tested compound via CAR nuclear receptor***.

Potency of each of the tested compounds, to transactivate p(ER6)$_3$-luc reporter construct by full-length human CAR, was assayed in transient transfection experiments in MZ-Hep1 cells. Quantification of luminescence of the compounds showed that compounds 21, 22, 23 and 24 activate efficiently p(ER6)3-luc reporter construct through CAR after 24 hours incubation in MZ-Hep1 cells. Furthermore, compounds 25 and CH3 also activate p(ER6)$_3$-luc but in a lower effect. These data show promising results for

further investigation and testing of the activation of CAR in vivo, even if gene reporter assay is a method that efficiently mimics the conditions found in living organisms.

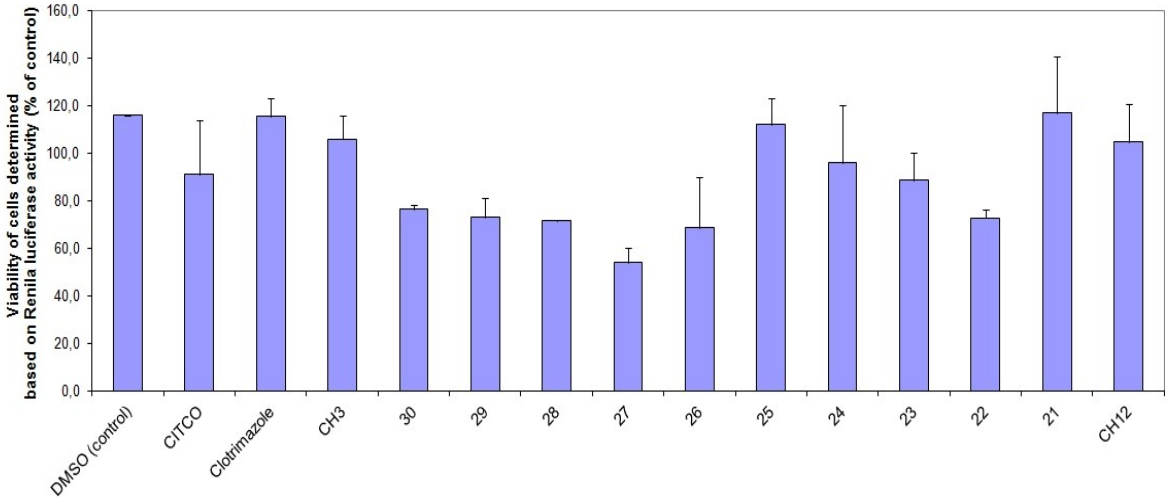


Fig. 17 ***Examination of cytotoxicity using Renilla luciferase activity of pRL-TK construct in MZ-Hep1 cells after treatment with tested compounds.***

MZ-Hep1 cells were transfected with a luciferase reporter construct $p(ER6)_3$-luc (150 ng/well), the expression plasmid encoding CAR (50 ng/well), and *Renilla reniformis* luciferase transfection control plasmid (pRL-TK, 30ng/well). 24 h later, cells were maintained in phenol red-free medium (200 µl) supplemented with 10% charcoal /dextran-stripped FBS with tested compounds for 24 h at the concentration of 30µM.

In the figure, data for Renilla luciferase activities are presented, which indicate potential cytotoxicity/antiproliferative activities of tested compounds over control (DMSO). CITCO, Clotrimazole and compounds CH3, 25, 24, 21 and CH12 indicate cells with normal proliferative activity. On the other hand, tested compounds 30, 29, 28, 27, 26, 23, 22 seem to be less proliferative.

7. Discussion

The human constitutive androstane receptor is one of the key regulators of xenobiotic and endobiotic metabolism. The constitutive androstane receptor (CAR), together with PXR, is major regulator of CYP gene expression. The unique properties of human CAR, such as the high constitutive activity and the complexity of signaling its numerous target genes in the liver attract attention of many researches. In addition, the lack of functional and predictive cell-based assays to study the properties of the receptor, limit the discovery of selective human CAR ligands. At presents, therefore, there are only few direct agonists and inverse agonists of the receptor. However, the compounds are either non-specific, toxic or with low affinity toward human CAR receptor (Swales and Negishi 2004, Pavek and Dvorak 2008, Maglich et al. 2009).

The present study aimed at discovering novel compounds that would extend the ligands as tools to study human CAR function. In the present screening study we have systematically analyzed the effect of 11 structurally diverse compounds on human CAR activation in two-hybrid assay. Activation of xenobiotic-sensing nuclear receptors can be measured with several high throughput in vitro methods including gene reporter assay with promoter sequences of target genes (such as CYP2B6) or using one- and two-hybrid assays. However, the ligand specificity of human CAR is still obscure, and this has been attributed mostly to lack of robustness of the assays and complexity of CAR signaling. The major reason is the high constitutive activity of CAR which impedes detection of human CAR ligands in cell-based reporter assays. Therefore, observed activation of a nuclear receptor using the methods does not always indicate significant induction of their target genes. This may be due to rapid metabolism of the inducing compounds or due to interference with other cellular processes that may affect CAR function.

In the diploma thesis, we identified 3 compounds that might be used as a chemical tool to study the biological functions of human CAR and also as a starting point for the development of new CAR ligands.

8. Conclusion

Since 1994, when CAR was first found many years of research activity have passed with numerous publications. Today, CAR is known to play a crucial role in the metabolism and biosynthesis of many endogenous compounds that mediate homeostasis of human organism such as bilirubin metabolism and bile acid regulation. The scientific community needs to take advantage of this critical characteristic of CAR , in order to find novel therapeutic ways to deal with various major diseases such as diabetes mellitus, chronic heart failure etc. However, many other features of CAR have not yet been revealed, which makes the continuous research even more intriguing. Furthermore, several newly synthesized ligands are tested nowadays for their potential in interacting with constitutive androstane receptor. Activation of CAR by any of these xenobiotics is being investigated and the binding ability with the receptor is measured. During my research I worked on 11 different compounds, each of them including a unique chemical compound. The graphs show promising results, indicating a number of the tested compounds binding effectively to CAR receptor and activating the expression of luciferase reporter construct in a high level. The environment that the compounds were tested is close to that in a living organism. So, the effective compounds may show great activity if introduced in a living organism that lefts to be seen in future studies.

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